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COLORADO TICK FEVER IN ALBERTA[1]

BY JOHN H. BROWN[2]

Abstract

The first record of Colorado tick fever virus in Alberta was made following the laboratory examination of 250 unfed *Dermacentor andersoni* ticks collected west of Calgary on May 11, 1953. On June 12, 1954, a further collection of 100 unfed ticks of the same species made in the same general area was also positive, and on June 28, 1954, a collection of 30 unfed *D. andersoni* made on pasture land at Brooks, about 165 miles east of the location of the previous positives, was also positive. Hence it would appear that Colorado tick fever virus is widespread in the *D. andersoni* population in Alberta. No human cases have been recognized in Alberta.

Colorado tick fever is the only virus disease of man in the Western Hemisphere known to be tick transmitted. It was first described by Becker (1) in 1930 who recognized it as a new disease and gave it the name of Colorado tick fever. In 1940 Topping, Cullyford, and Davis (3) concluded that the disease was probably associated with the bite of *Dermacentor andersoni.* In 1947 Florio and Stewart (2) demonstrated *D. andersoni* to be infected in nature and indicated that the infective agent may be transmitted through the egg to the next generation of ticks.

In man the disease is caused by the bite of an infected *D. andersoni* tick. The onset is usually sudden and clearly defined. The symptoms are headache, deep ocular pain, and lumbar backache; with anorexia, nausea, vomiting, photophobia, muscle pains, and hyperesthesia of the skin as a part of the syndrome. The temperature rises very rapidly and remains at 102 to 104° F. for about 48 hr. There is then a remission of the temperature for two or three days following which it rises again. The disease is never very serious but the period of convalescence and the amount of weakness is out of all proportion to the relatively short duration of the fever.

The first record of Colorado tick fever virus in Alberta was made in a communication from Dr. C. L. Larson, Director, Rocky Mountain Laboratory, Hamilton, Montana, reporting that 250 unfed *D. andersoni* ticks sent in by A. W. F. Banfield of the Canadian Wild Life Service were positive for this infection. The 250 ticks had been collected in the mountains west of Calgary on May 11, 1953.

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Contribution No. 55, Division of Entomology, Alberta Department of Public Health.
[2] *Provincial Entomologist.*

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On June 12, 1954, a collection of 100 *D. andersoni* also collected in the mountains west of Calgary was reported as being positive for Colorado tick fever. On June 28, 1954, a collection of 30 *D. andersoni* ticks taken on pasture land near the town of Brooks, Alberta, was also reported as being positive. These two collections were made by the Field Survey Crew of the Division of Entomology. The determinations for the presence of the infection were made at the Rocky Mountain Laboratory, Hamilton, Montana.

To date no human cases have been recognized in Alberta.

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EXTENT OF ICE FORMATION IN FROZEN TISSUES, AND A NEW METHOD FOR ITS MEASUREMENT[1]

By R. W. Salt[2]

Abstract

Use of the calorimetric and dilatometric methods for determination of ice in frozen tissues is criticized, and a method based on terminal melting points determined after various degrees of drying is proposed. The theoretical background of such a method lends support to it, and experimental work with gelatin is especially convincing. Use of the dehydration – melting point method on blood of *Loxostege sticticalis* gave results conforming in general to those obtained by other workers with calorimetric and dilatometric techniques, and also to those obtained with salt solutions. The amount of water that is bound is shown to be very low, as in mammalian, frog, and fish muscle. The possible influence of bound water in insect cold-hardiness is discussed and the conclusion is reached that it has little if any protective effect.

Introduction

Investigations by several workers have shown that ice formation in frozen tissues is incomplete at high subfreezing temperatures but increases rapidly with falling temperature. Mennie (5) found this to be true of fish muscle and gelatin, Scholander *et al.* (12) of *Chironomus* larvae and a lichen. Ditman *et al.* (3) found evidence of the same thing in six species of insects but interpreted the results differently. Theoretical considerations readily prove that extent of freezing in aqueous solutions varies with temperature between the freezing point and the eutectic temperature.

This paper deals with a new method for determining the extent of ice formation in tissues and breis, and discusses two alternative methods. In so doing the problem of bound water is raised and discussed in the light of present and past findings.

Criticism of Two Methods

Calorimetric Method

The calorimetric method, based on the difference in heat capacities or specific heats of ice and water, involves also a large element of uncertainty as to the thermal properties of the nonaqueous fraction. The practice of assigning mean values to quantities that vary appreciably with temperature changes is hazardous and hard to justify. The method may be suitable for large masses such as fish muscle, but does not seem to be sufficiently precise for entomological work.

Dilatometric Method

The dilatometric method is based on the expansion that takes place when water freezes to ice. It has been a popular method in many fields, but has disadvantages that have not always been recognized or adequately

[1] *Manuscript received May 18, 1955.*
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surmounted. Bouyoucos (1) used this method to measure the unfrozen water in soils of various types. In 1930, Sacharov (9) used it to determine the percentages of ice and water in frozen insects, apparently without modifying the technique in any essential way. Since the method measures the expansion of water on freezing, it should be obvious that no air or dissolved gases can be tolerated in the experimental material. Compressible gases would cushion the expansion, indicating less freezing than actually occurred. Bouyoucos evidently realized that air should be expelled, but undertook only partial measures to do so. He (1) states, "The whole mass was always shaken and stirred with a rod and also placed under a gentle force of suction to expell [sic] the air from the soil. This procedure seemed to exhaust the air quite completely from the soil; although some must still have remained in it which could not be seen. Various trials showed, however, that the results were not apparently affected by the presence of few air bubbles." Again (2) in 1936, "After the soil is allowed to stand for about 20 min. to absorb the water, the dilatometer is filled half-full with ligroin, stoppered, and the stem is connected to the suction force at the water faucet for a few seconds to remove the air from the soil. Care must be taken not to turn on a too strong suction force which will suck away the ligroin and water." It is apparent that these precautions are inadequate to remove gases dissolved in the water or air from the more finely textured and colloidal soils tested by Bouyoucos. In fact, the main finding of his work on a series of soils, i.e., that the more finely divided the soil the greater the proportion of water that did not freeze, could be explained by a progressively greater failure to remove air from the samples as the soil texture became finer and more colloidal. His reference to the lack of influence of a few air bubbles is certainly open to question. Scholander *et al.* (12) have pointed out that "The freezing out of gases in the commonly used dilatometric methods is a source of error, which has been largely overlooked".

In insects there are two distinct sources of gases, the tracheal system and body moisture. The latter, upon freezing, forces dissolved gases out of solution, a process readily seen in the freezing of tapwater. The dissolved gases occupy no measurable volume, but once frozen out of solution they occupy space. In a dilatometer this would increase the observed expansion, an effect opposite to the cushioning one provided by gases already present in the gaseous phase. As soon as a gas is frozen out of solution and has added its volume to the system, it is then in a position to be compressed by further crystallization and to tend to reduce the volume. Thus we have two opposing effects that make accurate measurement in a dilatometer difficult to conceive, let alone execute.

The writer attempted to eliminate the tracheal air and most of the dissolved gases in his experimental material, which included larvae of Hymenoptera, Lepidoptera, and Diptera, by using a modification of Wigglesworth's tracheal impregnation technique (13). The larvae were suspended above light paraffin oil in a vacuum flask; after about a minute of evacuation by a Hyvac pump

the larvae were submerged and the vacuum suddenly released, filling the tracheae with oil. Similar oil, colored red with Sudan III, was used in the dilatometer. Theoretically most of the dissolved gases also escaped; however, on several occasions bubbles of gas were observed after insects frozen in the dilatometer were thawed. Gas elimination was incomplete even when evacuation was prolonged or repeated.

The data obtained on various larvae gave curves similar to those obtained dilatometrically by Moran (6) on gelatin gels. Unfortunately the curves could not be depended upon to coincide as the temperature was raised or lowered, although some fitted reasonably well. Moran also had difficulty in this regard, for one of his two published curves shows perfect coincidence on the subfreezing end but not on the unfrozen section, whereas of the other curve the opposite is true. Moran notes that this has been "observed for different substances by other investigators, notably Foote and Saxton (1916-17) and is ascribed to the rupture of capillaries in the freezing process, with consequent increase in the volume of the system as a whole". Although this may be true, the writer's investigations do not particularly support it, as the volume of the system decreased as often as it increased. For example, without being removed from the dilatometer, larvae were frozen and thawed repeatedly, either during a few hours or slowly over a period of weeks, to allow ample time for equilibration. The curve shifted both upward and downward (increased and decreased volume) in both the frozen and unfrozen states. Ample time was allowed for possible "frozen out" gases to be redissolved, but as often as not the change was in the wrong direction.

For these reasons it was felt that the dilatometric method was not sufficiently accurate for entomological work. It did indicate the general picture, however, producing curves similar to those that Moran (6) obtained with gelatin. The same type of curve resulted from an entirely different method, the density–flotation method, developed by Scholander *et al.* (12). These authors used the difference in density of ice and water as a basis for calculating the amounts of ice and water, from 0° C. to −35° C., in *Chironomus* larvae. These larvae, however, normally have no air-filled tracheal system and so represent a special case. Replacing tracheal air with oil, as already described, would not be a permissible modification of this method for terrestrial insects. Scholander *et al.* (12) recognized the fact that dissolved gases would invalidate their results, and as a test they rapidly thawed frozen larvae in warm water under a microscope. Although thawed *Chironomus* larvae are transparent, air bubbles were never observed. This is indeed remarkable. In any case, however, the method is of limited value since most insects have air-filled tracheae.

Dehydration - Melting Point Method

Theoretical Considerations

The method finally used was developed by the writer on theoretical grounds alone, and arose out of a consideration of the behavior of salt solutions and

gelatin gels. These may be taken as representative, in simple form, of the two classes of substances likely to affect the freezing of biological tissues—low molecular weight solutes such as salts and sugars, and high molecular weight fractions such as proteins. The technique of the method, referred to as the dehydration – melting point method, is given after exposition of the theoretical bases.

Fig. 1 depicts the relationship between sodium chloride concentration and the freezing point depression of water. The ordinates are concentrations of the solute, but in closer analogy to biological terminology they are also converted to concentrations of solvent, or moisture content. The abscissae represent depressions of the freezing point of the pure solvent, which is of course the only portion that actually freezes. Strictly speaking, solutions have no freezing and melting *points*, but only freezing and melting *ranges*. It is convenient, however, to speak of the highest temperature at which ice can be formed in a solution as its freezing point. It is also the melting point, for at this temperature the last crystal of ice will disappear upon melting. In Fig. 1, therefore, there can be no more than a trace of ice at any point *on* the curve.

If the basic data of Fig. 1 are converted to a different form an interesting result is obtained. If a 1% NaCl solution, chosen because it is approximately isosmotic with insect tissues, is gradually dried, the curve in Fig. 2, *A*, represents the result. The ordinates represent the percentage of the original

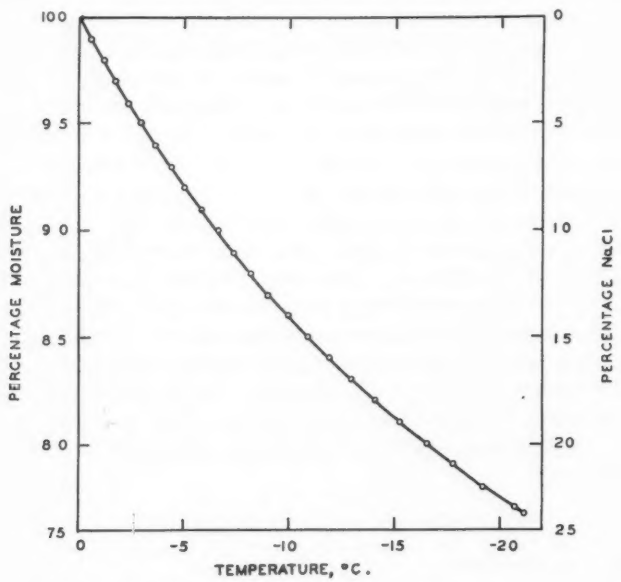

FIG. 1. Freezing point depression of sodium chloride in aqueous solution (From data in Lange, 1952 (4)).

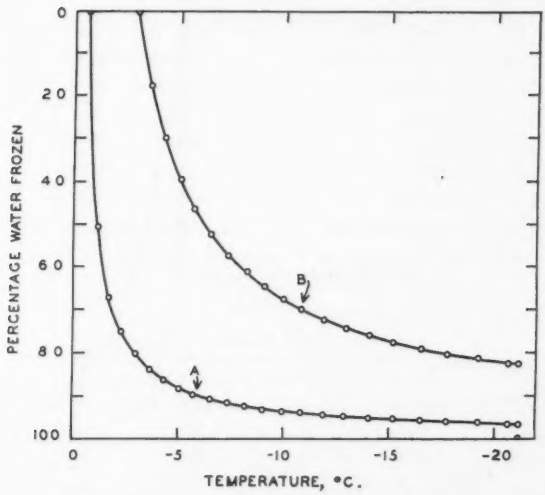

FIG. 2. Relation between temperature and ice formation in sodium chloride solutions of 1% (*A*) and 5% (*B*) concentration (From data in Lange, 1952 (4)).

amount of water that has been removed. It makes no difference how the water is removed as long as it is no longer present as a solvent. Freezing accomplishes this automatically as the temperature is lowered, until the eutectic temperature is reached. Hence the ordinates in Fig. 2, *A*, represent the percentage of water frozen in a 1% NaCl solution. Or, knowing the concentrations of the unfrozen solution, one can calculate exactly the ratio of ice to water at any temperature.

The influence of solute concentration is exemplified in Fig. 2, *B*, by a 5% NaCl solution. This differs from the 1% curve in three ways: the freezing point is lower, the rate of ice formation per degree fall in temperature is lower, and the percentage of ice at a given temperature is lower. If a 1% solution is cooled to −5.03° C., 88.4% of its water freezes, and the unfrozen solution has a concentration of 8%. If a 5% solution is cooled to the same temperature, the unfrozen solution is also 8% but only 39.5% of the original water has frozen.

By analogy, a tissue with a greater freezing point depression will be less completely frozen at a given temperature than one with a higher freezing point. This will apply to any aqueous solution, but the effect will be inversely proportional to the molecular weight of the solute(s) (making due allowance for ionization); that of proteins will be insignificant.

It is therefore remarkable that gelatin produces curves similar to those in Fig. 2, when plotted in the same way. Those in Fig. 3, *A* and *B*, are drawn from Moran's (6) data on gelatin gels of 12 and 40%, and Fig. 3, *C*, is drawn from the data of Mennie (5) on a 40.5% gelatin gel. The closeness of curves *B* and *C* speaks well for the accuracy of the two methods. Moran removed

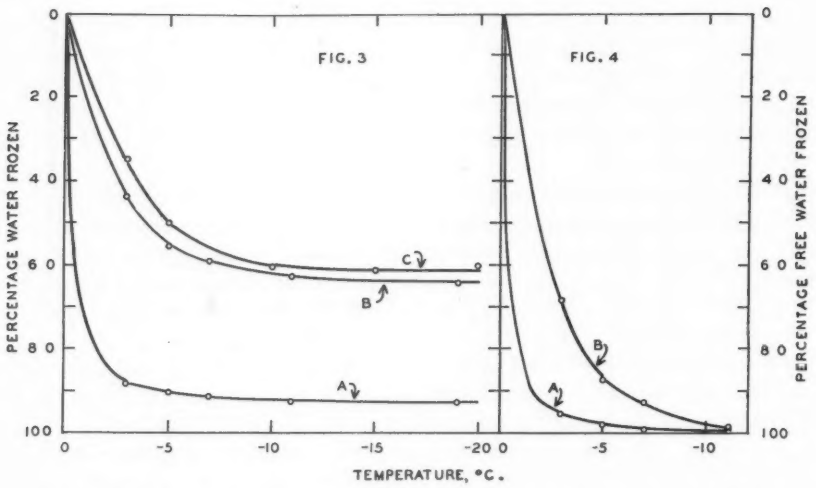

FIG. 3. Relation between temperature and ice formation in gelatin gels. *A*, 12% gel; *B*, 40% gel (data from Moran, 1926 (6)); *C*, 40.5% gel (data from Mennie, 1932 (5)).

FIG. 4. Relation between temperature and the freezing of free water in gelatin gels. *A*, 12% gel; *B*, 40% gel. (Data from Moran, 1926 (6)).

the shells of pure ice from disks of gelatin and determined the concentration of the unfrozen cores; Mennie calculated his values from heat capacity data by means of an equation. The freezing point depression of these gels is, of course, very small (about 0.007° C. for a 12% gel, 0.023° C. for a 40% gel). Their similarity to the NaCl curves lies rather in the effect of gelatin concentrations on the rate and extent of ice formation. Moran found that, when gels of any concentration between 12% and 40% were frozen and held for a time at a subzero temperature, the unfrozen portion of the gel was always of the same concentration at a given temperature, regardless of the original concentration. Thus the curves in Figs. 2 and 3 are similar in that at a given temperature the unfrozen portion has a fixed concentration, irrespective of initial concentration. The difference is that in the salt solution the unfrozen solution is still at or above its freezing point, whereas the gelatin solution is below its freezing point. Why, then does it not freeze ?

The investigations of many workers indicate that gelatin binds water and that this water will not freeze, at least at temperatures that freeze free water. Moran (6) found that gelatin gels over 65% would not freeze, even in liquid air, and other experiments of his also indicated that all of the water was bound when the gels reached this concentration. If we accept this figure as a close approximation we can then re-plot Fig. 3 with free water rather than total water as the basis. When this is done (Fig. 4), total freezing of free water is approached at fairly high temperatures, but still far from the freezing point. Is this due to the presence of low molecular weight impurities ? Moran's gelatin had an ash content of 0.05%, Mennie's 2.8%. The question is

answered by calculating the equivalent concentrations of NaCl that would produce freezing point depressions as a Fig. 4 (Table I). Values below −7° C. are omitted because the proximity to complete freezing lowers the accuracy of the calculations. If the curves were in fact caused by impurities, the NaCl equivalents listed in Table I would be the same at each temperature level (cf. Fig. 2), which they are not. Moreover, the values for each gel should increase as the temperature is lowered, whereas the opposite was observed. With this possibility ruled out, then the logical explanation seems to be that the amount of bound water was not constant, but varied with temperature.

TABLE I

CONCENTRATIONS OF NaCl SOLUTIONS PRODUCING THE FREEZING POINT DEPRESSIONS OF FIG. 4. DATA CALCULATED FROM MORAN (1926)

	Equivalent NaCl solution	
Temperature, ° C.	12% gel	40% gel
− 3	0.22	1.58
− 5	0.15	1.02
− 7	0.11	0.78

If this is accepted, then all of the free water in a pure gelatin gel freezes at the freezing point of the initial gel, slightly below 0° C. Lowering the temperature, however, liberates some bound water, which thereupon freezes. The curves in Fig. 4 are therefore invalid, but those in Fig. 3 are valid and useful for determining the relationship of water binding to temperature, particularly when converted back to the form in which Moran (6) originally presented them (Fig. 5).

Some explanation is first needed regarding the dilute gels. Moran found that disks (15 × 3 mm.) of gels up to 12% froze throughout, whereas in gels of 12% and over a shell of pure ice formed around the disk. In the latter gels the unfrozen cores reached an equilibrium concentration that was always the same at a given temperature. These are the values plotted in the upper section of Fig. 5. The writer has extrapolated the curve to cut the axis at the 12% level because of the different freezing behavior of gels above and below this concentration (6). Moran's observations warrant the extrapolation down to 12%, but not below that figure. If the change in freezing behavior means that there is no bound water in gels weaker than 12%, then the curve should cut the axis at the freezing point of the gel, just below 0° C. This is merely speculation, of course.

Even though the freezing point is so close to 0° C., no freezing will take place in a gel more concentrated than 12% until a lower temperature is reached, as shown in Fig. 5. For example, if we slowly cool a 54.3% gel to −5° C. there will be present a shell of pure ice and a core of unfrozen gel with a concentration of 60.1% (6). If this is slowly warmed, ice melts and enters the core to dilute it. When the temperature reaches −3° C. the last bit of

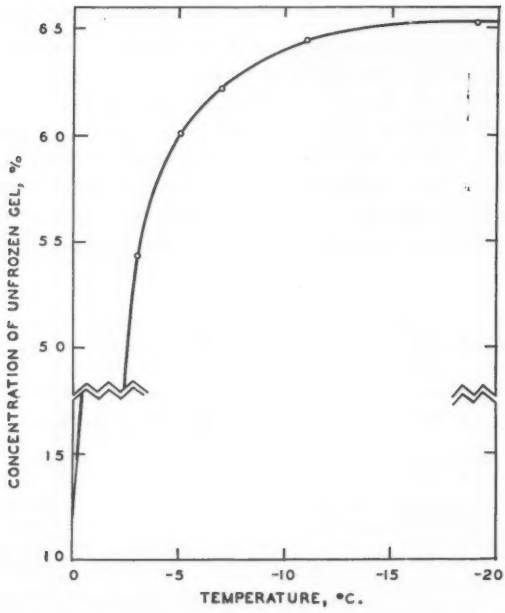

FIG. 5. Relation between temperature and the unfrozen portion of gelatin gels. Upper section of curve from Moran, 1926 (6). Extrapolation to 12% level by the author.

ice melts and the gel has been diluted back to its original concentration. It is impossible, therefore, to produce freezing in a 54.3% gel at a temperature above −3° C. Above this temperature all of the water is bound, and in addition there is a potential to bind a little more; below −3°, some water leaves the bound state and freezes. All of this is in accord with the micellar theory, in which the strength of binding decreases as the distance from the micelle increases.

Experimentally Established Considerations

We have thus far observed the relationship between subfreezing temperatures and ice formation in two relatively simple systems. Although it is a far jump to biological tissues, the reactions are made more understandable by what happens to salt and gelatin solutions. The published curves of Scholander *et al.* (12) for *Chironomus* larvae and of Ditman *et al.* (3) for the European corn borer, codling moth larvae, the cornstalk borer, the corn earworm, squash bug adults, and Mexican bean beetle adults resemble Fig. 2, *A*, very strongly, modified perhaps by a small percentage of bound water. Feeling that the methods used by these authors, the density–flotation method and the calorimetric method respectively, were perhaps not sufficiently accurate, the writer developed a method that appeared to be new. Later it was discovered that, although the method itself was new, its basis was firmly

established by Moran (7) in 1929. Thus Moran's work, which contributed appreciably to the theoretical considerations discussed in the preceding section, also supplies an excellent experimental basis for the procedures to be outlined later.

Moran (7) compared the effects of water loss by drying and by freezing (determining the conversion of water to ice dilatometrically) in frog muscles. When water was removed by either drying or freezing, recovery and the responses of muscles to stimuli were the same. The original state of the muscle was completely recovered by restoring water where the loss had been less than 40%; between 40 and 78% water loss, physiological activity decreased with time of exposure but the physicochemical properties were unaltered; beyond 78% loss there was no recovery. The critical value of 78% water removal corresponded to freezing at −2° C., which was demonstrated experimentally to be the critical temperature for survival after freezing. Thus Moran showed that injury to muscle by freezing was caused by dehydration, and the interchangeability of freezing and drying, as used in the following method, was established as valid.

Procedure and Limitations

It has been shown that a 1% NaCl solution can be dehydrated by either evaporation or freezing to produce the curve in Fig. 2, *A*; the important thing is to remove solvent. The writer therefore exposed insect blood to various degrees of dehydration by means of a dry air stream, measured the melting points of the residues, and plotted the data in the same form as Fig. 2. Typical results of a number of tests made with blood of *Loxostege sticticalis* (L.) are shown in Fig. 6. Its similarity to those of Scholander *et al.* (12) and Ditman

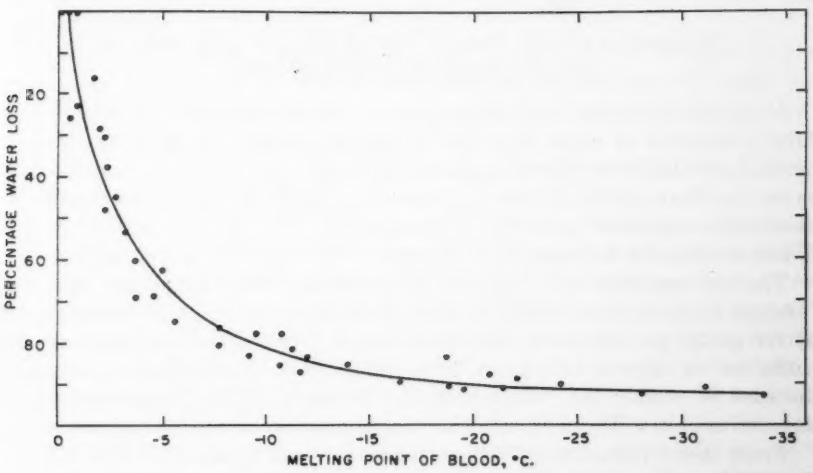

FIG. 6. Melting points of samples of blood from *Loxostege sticticalis* larvae in relation to water loss. Loss equivalent to water frozen in undried blood.

et al. (3) indicates that all three methods are adequate. Its similarity to Fig. 2, *A*, indicates that the low molecular weight solutes are dominant and that bound water is not very plentiful. In fact, were it not that ionic interactions and eutectic temperatures are unknown quantities, the entire curve might be interpreted on an osmotic basis. Moran (8) found that there was very little bound water (less than 6%) in mammalian muscle.

It may be objected that drying the blood by evaporation, even though accomplished rapidly by a stream of dry air, had the effect of releasing bound water to augment the decreasing free water. If so, the transfer must be small, since the results are so like those produced by the density–flotation method (12) and the calorimetric method (3), in which there was no possibility of such transfer. The same may be said of objections based on other changes in the blood during drying, such as oxidation. Minor effects undoubtedly exist, but it is the general features of the curve that are important, rather than exact location.

The dehydration – melting point method is better adapted to fluid tissues like blood than to more solid tissues or whole insects. It could be used on breis if they are kept homogeneous during dehydration, and if the end point of melting can be detected, visually or otherwise. The chief source of error in the method lies in differential drying; the melting point recorded will be that of the most dilute portion of the sample, but the weight from which the water loss is calculated will be that of the entire sample.

Although the method is simple, it has limited applicability; nevertheless, its theoretical aspects are of great importance. Its corroboration of the general results obtained with the calorimetric and dilatometric methods lends credence to the basic soundness of the three methods and, indirectly, of the principles derived through their use.

Discussion of Ice Formation in Insects and Role of Bound Water in Cold-Hardiness

A striking feature of the temperature – ice formation relationship is the large proportion of water frozen to ice during the first degree or two drop in temperature below the freezing point of the fresh tissue. Also very noticeable is the fact that additional freezing diminishes rapidly as the temperature falls, resulting in a gradual approach to a level not far from complete crystallization. What significance do these features have in the cold-hardiness of insects?

The vast majority of insects die if frozen, but there are others that can tolerate ice formation in their tissues. The extent to which ice forms in the latter group has not been adequately investigated, but it is known to be sufficient to impart brittleness. No satisfactory hypothesis has been put forward to explain the different effect of freezing in the two groups, which for convenience will be designated as freezing-susceptible and freezing-resistant.

Fresh insect tissues usually have freezing points between 0° and −3° C., but they readily supercool to lower temperatures. In fact, it is very difficult to induce freezing in an unharmed insect without supercooling it a few degrees.

Supercooling can be reduced greatly by surface moisture, whether this be secreted, excreted, or environmental, but it will not be eliminated. If an insect freezes under natural conditions it will be at a temperature at least two or three degrees below its freezing point, and a large portion of its body moisture will freeze. When a freezing-susceptible insect undergoes this near-minimal treatment it is not killed outright, but dies after a period of subnormal existence. This has been observed by the writer to be true of a number of species. Normally, however, a dry hibernating insect will supercool to a characteristic temperature range before freezing. Supercooling protection of 15° to 20°, is only average; in some forms such as eggs of *Mantis religiosa* L. it attains more than 40° C. (10). Thus an average insect of the type not tolerant of freezing, upon reaching its limit of supercooling, will have over 80% of its total water changed to ice in a matter of seconds; but whether supercooling is great or small the insect is killed by ice formation, and whatever bound water content it possesses is of no avail once freezing starts. The only time when bound water can conceivably protect such an insect is before freezing, by lowering its supercooling point. But this has never been demonstrated, either experimentally or on theoretical grounds, and in the light of present evidence, appears unlikely to be appreciable (11). Since a high percentage of the body water crystallizes at the undercooling point, and some of the remainder is a concentrated solution mixture, the percentage of bound water cannot be high. In this respect insect blood resembles mammalian muscle (8).

If bound water does not appear to contribute appreciably to the cold-hardiness of freezing-susceptible insects, does it offer an explanation for the ability of the freezing-resistant group to tolerate freezing? The writer has no experimental evidence to offer on this subject, but a little speculation may be permissible.

Gelatin binds water, and other hydrophilic colloids are considered to do likewise. The water so bound is usually considered nonsolvent water, and indeed this has been used as the basis of a method for measuring bound water. However, the presence of salts and other solutes probably reduces the amount of bound water (8); the effect of increasing solute concentration does not appear to be adequately known.

We have seen that the percentage of bound water decreases with falling temperature; the free water thus converted becomes available for freezing or for diluting the unfrozen portion. The bulk of the bound water, however, remains unfrozen. The protection that this unfrozen water gives to an insect, for example, is debatable. If one uses total body moisture as a basis, then the progress of ice formation is that delineated in Fig. 3, where the percentage of unfrozen water appears safely high in concentrated gels. But the injury, if any, is accomplished by the freezing fraction of the water, and it is this fraction that is important. Its ice formation – temperature curve is like those of Fig. 2, with initial concentration determining its position. If ice formation is the only source of danger, then the more concentrated the solution the

greater the safety, the curve moving upwards and to the right. Increasing solute concentration may eventually become injurious of itself. If, in acquiring cold-hardiness, an insect converts free water to bound water and consequently increases the solute concentration in the free water, the protection should be credited to the solution, not to the bound water. The same effect could be attained, if this reasoning is correct, by removing water by evaporation or by increasing the solute content through chemical reaction. Hence the role of bound water in cold-hardiness is still not demonstrated or explained.

It has been suggested above that bound water cannot protect a freezing-susceptible insect, except possibly by depressing the undercooling point to a very slight extent. The writer shows elsewhere (11) that the loss of water by such insects has a negligible effect on their hardiness. As they become drier their undercooling points fall very slightly throughout a large range of moisture content. Undercooling then increases more and more rapidly with further drying, but unfortunately this is of no value to the insect for by this time it has already died from desiccation. This is further evidence that bound water plays little or no part in the cold-hardiness of freezing-susceptible insects.

In summary, it appears that neither bound water nor solute concentration does much to increase the cold-hardiness of freezing-susceptible insects, although solute concentration may be a major factor in giving the insect the cold-hardiness it already possesses. In freezing-resistant insects, bound water and solute concentration may together give the insect its existing cold-hardiness, but the solute concentration is the factor that really confers it. In a sense the bound water is not a part of the body moisture, and acts only negatively by removing itself from the system, neither freezing nor acting as solvent. Evaporation of free water produces the same result.

Acknowledgment

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THE SELECTION BY CERTAIN SMALL MAMMALS OF DEAD, PARASITIZED, AND HEALTHY PREPUPAE OF THE EUROPEAN PINE SAWFLY, *NEODIPRION SERTIFER* (GEOFF.)[1]

By C. S. Holling[2]

Abstract

Caged *Sorex cinereus cinereus* Kerr, *Blarina brevicauda talpoides* Gapper, and *Peromyscus maniculatus bairdii* Hoy and Kenicott opened more cocoons containing healthy, living sawfly prepupae than ones containing prepupae attacked by fungus. In the field and in the laboratory, *S.c.cinereus* opened more healthy cocoons than cocoons containing parasites. The reverse appeared to be true for *B.b.talpoides*. *P.m.bairdii* opened equal numbers of healthy and parasitized cocoons. The more insectivorous animals exhibited the greatest selective ability. Pretreatment to cocoons of one category affected the selective ability. Pretreating *P.m.bairdii* to fungus cocoons lowered the ability to select healthy cocoons over fungus cocoons. Pretreating animals to healthy cocoons raised the selective ability. Selection occurred in the digging, removing, opening, and eating phases in the search for cocoons. The closer the phase to the consummatory action, the greater was the degree of selection. The selective ability in the first three phases was acquired with experience. In the final, eating, phase it was innate.

Introduction

Small mammal predation affects populations of many forest insects. This is particularly true in the case of those sawflies that spin cocoons in the ground where they are exposed to mammal predation. In 1928, Graham (2) showed that small mammals are important in controlling populations of the larch sawfly *Pristiphora erichsonii* (Htg.). Subsequently Morris (8) showed that predation by small mammals was a very important factor affecting cocoon populations of the European spruce sawfly *Gilpinia hercyniae* (Htg.). The problem discussed in this paper arose from an investigation of the effect of small mammal predation in controlling populations of the European pine sawfly *Neodiprion sertifer* (Geoff.). These sawfly attack Scots, jack, and red pines, and have achieved epidemic proportions in the Scots pine plantations of southwestern Ontario. In samples of cocoons collected from the forest floor, up to 70% of the cocoons were opened by small mammals. This statement of their controlling effect, however, is imprecise.

In order to assess precisely the importance of small mammal predation in controlling sawfly populations, the reactions of the mammal predators to various kinds of cocoons must be considered. Normally cocoons in the ground can be categorized as to empty or sound. Some of the latter contain living healthy sawflies while others contain sawflies attacked by parasites or disease. In addition, with certain sawflies, there are male and female cocoons. It is important to know if the mammals can differentiate between these categories. For example, if small mammals avoided opening cocoons containing parasitized sawflies, their *effective* control would be considerably greater.

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Although it is well known that animals in general are discriminating in their choice of food, little is known about the selective ability of small mammals. The information available has been principally derived from the analysis of the stomach contents of small mammals. The data, however, cannot be precisely interpreted since the food is finely masticated and undigestible parts such as insect sclerites and seed coats are partially rejected. In addition, the availability of food items might completely mask apparent food preferences. According to Jameson (6) and Hamilton (5) *Peromyscus* eat seeds, arthropods, fruits, leaves, and fungi in decreasing order of abundance. Similar studies (3, 4, 7) of the shrews *Blarina* and *Sorex* indicate that arthropods, particularly insects, make up the bulk of the food, although worms and molluscs are also eaten. Vegetable food is of minor importance. More precise information has been published by Morris (9) on the basis of experiments conducted with caged animals. He has shown that several species of small mammals open more sound than empty cocoons of *G. hercyniae.*

It was not known, however, if this selective ability extends to other categories of cocoons. The purpose of the study presented in this paper therefore was to investigate the selective ability of certain small mammals in relation to cocoons containing healthy, diseased, and parasitized prepupae of the sawfly *Neodiprion sertifer* (Geoff.). In addition, the development of the behavior was investigated. The mechanisms of selection and the reactions of the small mammals to male and female cocoons will be discussed in a later paper.

Materials and Methods

The deermouse *Peromyscus maniculatus bairdii* Hoy and Kenicott and the shrews *Blarina brevicauda talpoides* Gapper and *Sorex cinereus cinereus* Kerr were used in the experiments to test selective ability. Extensive trapping every month for two summers with Sherman live traps, bucket traps, and Victor snap-back traps indicated that these three species were the common small mammals residing in the sawfly-infested Scots pine plantations of southwestern Ontario. The deer mice used in laboratory experiments were trapped using Sherman live traps baited with peanut butter and oatmeal while the shrews were captured in bucket traps provided with canned meat prepared as dog food. All animals were kept in $12 \times 12 \times 12$ in. cages with glass fronts and screen tops and were provided with nesting cotton and a continuous supply of water. The deer mice were fed with fox chow pellets while the shrews were provided with the canned dog food. All animals survived well on these diets.

Feeding behavior was studied using the sawfly *N. sertifer* as food. This insect overwinters in the egg stage and hatches in early May. The larvae feed on the trees until the early part of June when they drop to the ground and spin cocoons in the litter and duff under the trees. Emergence from the cocoon commences during the latter part of September. Since most of the small mammal predation occurs after larval drop and before adult emergence,

it is important that the feeding behavior of the small mammals in relation to sawfly cocoons should be studied.

The sawfly cocoons were collected in southwestern Ontario in Scots pine plantations heavily infested with the sawfly. Food selection was studied using categories of cocoons grouped as follows:

(1) cocoons containing healthy, living sawfly prepupae vs. cocoons containing dead prepupae that had been attacked by fungus.

(2) cocoons containing healthy, living sawfly prepupae vs. cocoons containing dead sawfly prepupae and living parasites. Approximately 80% of the parasites were *Exenterus canadensis* Prov. The remainder were composed principally of *Euceros frigidus* (Cress.) and *Hemipenthes sinuosa* (Wd.).*

Where brevity is desired cocoons containing healthy, living sawfly prepupae will be termed "healthy" cocoons, cocoons containing dead prepupae attacked by fungus will be termed "fungus" cocoons, and cocoons containing dead prepupae and living parasites will be termed "parasitized" cocoons.

Healthy, fungus, and parasitized cocoons were segregated by X-ray analysis using a General Electric X-ray Grain Inspection unit.† An exposure of 1.5 min. at 20 kv. and 5 ma. produced negatives from which cocoons could readily be identified as healthy, fungus, or parasitized (Fig. 1). The cocoons were prepared for X-raying by rolling them in 14 in. long cigarette papers with a V-master cigarette roller. Each picture accommodated approximately 60 of these rolls. By numbering each roll and the X-ray negative each cocoon could be identified for later segregation. With practice even cocoons containing very young parasite larvae could be identified. As a check of the accuracy of the method, 150 cocoons were identified from an X-ray photograph and then opened for positive identification. The agreement was 100%.

In the selection experiments the two categories of cocoons presented were each marked with an india ink stroke 3 mm. long. The mark ran parallel to the longitudinal axis of cocoons of one category and perpendicular to the axis of cocoons of the other.

Data on the selection of cocoons were obtained from experiments with caged animals and from the analysis of cocoons collected from the forest floor. The experimental cages used were identical with the ones in which the animals were normally kept. The floor of the cage was covered with a 4 cm. layer of fine washed quartz sand. After the animals were familiarized with the

* *Mr. K. J. Griffiths of the Forest Insect Laboratory, Sault Ste. Marie, Ontario, kindly provided information on the species and relative numbers of parasities present in the area where cocoons were collected.*

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FIG. 1. X-ray of *N. sertifer* cocoons showing: (*a*) parasitized cocoons, (*b*) fungus cocoons, (*c*) healthy cocoons.

FIG. 2. *N. sertifer* cocoons showing from left to right cocoons opened by *S.c.cinereus*, cocoons opened by *B.b.talpoides*, and cocoons opened by *P.m.bairdii*.

PLATE I

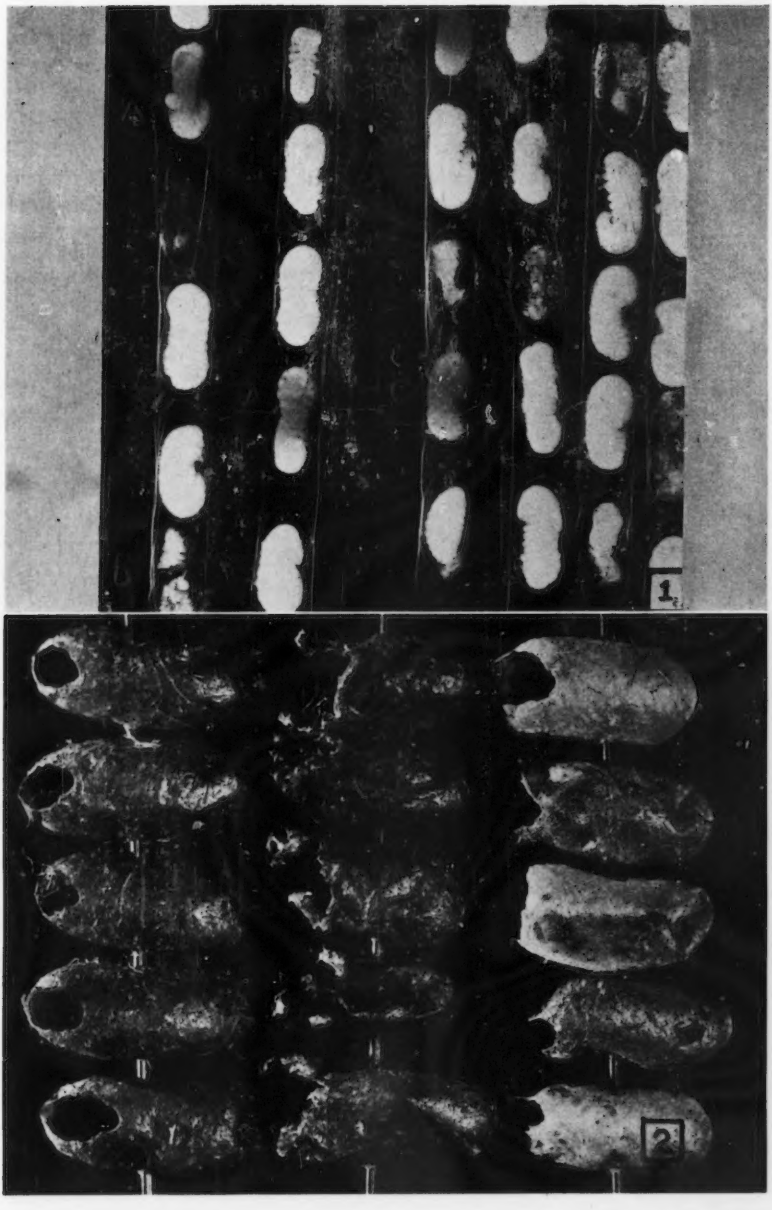

PLATE II

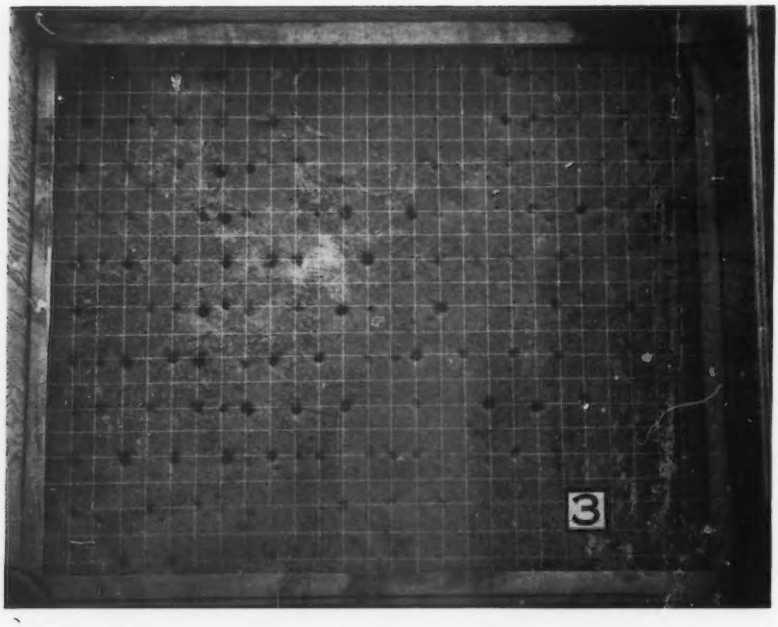

FIG. 3. Photograph of cage used in grid experiments. Cocoons were buried where the lines intersect. The holes were dug by one *S.c.cinereus* during a 24 hr. period.

experimental cage for one week they were tested individually for selective ability for at least seven consecutive experiments. At the beginning of each experiment equal numbers of two categories of cocoons were mixed thoroughly and were buried under the layer of sand. The animal remained in the cage for 24 hr. At the end of the experiment the number of cocoons of each category opened and unopened were counted and a new group was provided to begin another experiment. At all times water and additional food, fox chow for the deer mice and dog food for the shrews, was present. When the selection of healthy vs. fungus cocoons and healthy vs. parasitized cocoons was tested, each animal was provided with either male or female cocoons of the two categories so that selection would not be masked by a "preference" for one of the sexes.

Selection is numerically indicated by an index of selection adopted from Dice (1):

$$I = \frac{Ba - Ab}{Ba + Ab}$$

where A = the number of cocoons of the first category provided,
a = the number of cocoons of the first category opened,
B = the number of cocoons of the second category provided,
b = the number of cocoons of the second category opened.

When equal numbers of each category are provided the formula reduces to

$$I = \frac{a - b}{a + b}.$$

The index ranges from -1.0 when $a = 0$, through 0.0 when $a = b$, to 1.0 when $b = 0$. The first category was arbitrarily assigned to healthy cocoons and the second to fungus and parasitized cocoons. Between two categories, the differences in the numbers of cocoons opened were tested for significance using the chi-square test for a 2×2 contingency table.

Information on cocoon selection was also obtained from the analysis of cocoons collected from the forest floor. The cocoon sampling technique was essentially the same as that employed by Prebble (13) for the European spruce sawfly. A large number of Scots pine trees (at least 100) were selected and marked throughout a plantation. Two 1-ft. square quadrats were randomly selected beneath the crown of each tree and the litter and duff were removed. This material was then sifted through screens which separated the cocoons from the litter and duff. The cocoons were then analyzed into the following groups: healthy cocoons, fungus cocoons, parasitized cocoons, cocoons opened by small mammals, and other empty cocoons.

Results

The Selection of Cocoons

Selective ability was investigated using animals with two types of feeding history. The animals of one group had just been captured and thus had been exposed to a variety of natural occurring foods, including cocoons. The

animals of the other group had either been born and raised in captivity (*P.m.bairdii*) or had been isolated from cocoons for at least five months (*S.c.cinereus* and *B.b. talpoides*). The deer mice therefore had received only fox chow biscuits as food and the shrews had received only canned meat. Experiments with the former group were conducted out of doors immediately after their capture, in cages set up in a Scots pine plantation. Experiments with the latter were conducted in a constant temperature room kept at approximately 15° C.

In order to simulate the feeding history of animals in the field, before testing, the animals of the second group were provided with 100 healthy cocoons each night for seven nights. For four subsequent nights they were provided with 100 cocoons of each of the two categories under study. This pretreatment was immediately followed by the actual selection experiments. In the experiments conducted under field conditions unequal numbers of each category were provided because of an inadequate supply of cocoons. In the experiments conducted under laboratory conditions equal numbers of cocoons of each category were provided.

Selection of Healthy Cocoons vs. Fungus Cocoons

The grouped results of cage experiments using *S.c.cinereus*, *B.b.talpoides*, and *P.m.bairdii* provided with healthy cocoons and fungus cocoons are presented in Table I. Each animal opened more healthy cocoons than fungus cocoons; that is, the indices of selection were positive. In addition, the indices in each experiment were always positive and the range of these indices was not great. The chi-square test applied to the grouped data indicated that the probability (designated as P in the Table) of this selection occurring by chance alone was less than one per cent.

Selection of Healthy Cocoons vs. Parasitized Cocoons

The results of experiments studying the ability of the three species of small mammals to differentiate between healthy and parasitized cocoons are listed in Table II. Both the shrews exhibited a significant degree of selection while the deer mice did not. *S.c.cinereus* opened more healthy than parasitized cocoons, a positive index being obtained in every experiment. In contrast, *B.b.talpoides* opened more parasitized than healthy cocoons, a negative index being obtained in every experiment.

If the sign of the index of selection is ignored, the numerical values of the indices provide an indication of the degree of selective ability of the three species. There is a gradation in the degree of selection from *S.c.cinereus*, through *B.b.talpoides* to *P.m.bairdii*. The same gradation can be seen in Table I where the selection of healthy cocoons vs. fungus cocoons is demonstrated. This is similar to the gradation in insectivorous habit of the species, reported by Hamilton (3, 5), Jameson (6), and Morris (9). *S.cinereus* is highly insectivorous, *B.brevicauda* less so, while *P.maniculatus* is omnivorous. The more insectivorous the species the greater is its selective ability. Morris

TABLE I

SELECTION OF *N. sertifer* COCOONS BY CAGED SMALL MAMMALS. HEALTHY COCOONS VS. FUNGUS COCOONS

Species	No. animals	Exptl. conditions	No. expts.	No. cocoons provided		No. cocoons opened		P	Index of selection and range between experiments
				Healthy	Fungus	Healthy	Fungus		
S.c.cinereus	1	Field	3	280	187	173	3	< 1%	0.949 (0.895 to 1.00)
	1	Laboratory	3	376	376	229	2	< 1%	0.982 (0.978 to 1.00)
B.b.talpoides	2	Field	5	497	168	304	20	< 1%	0.674 (0.575 to 0.833)
P.m.bairdii	2	Field	3	291	191	250	48	< 1%	0.547 (0.430 to 0.660)
	12	Laboratory	53	5300	5300	4098	439	< 1%	0.807 (0.614 to 1.00)

TABLE II

SELECTION OF *N. sertifer* COCOONS BY CAGED SMALL MAMMALS. HEALTHY COCOONS VS. PARASITIZED COCOONS

Species	No. animals	Exptl. conditions	No. expts.	No. cocoons provided		No. cocoons opened		P	Index of selection and range between experiments
				Healthy	Par.	Healthy	Par.		
Sc.c.cinereus	1	Field	35	2456	1186	1122	151	< 1%	0.565 (0.423 to 0.615)
	1	Laboratory	7	634	634	289	149	< 1%	0.320 (0.146 to 0.466)
B.b.talpoides	3	Laboratory	23	2300	2300	761	1092	< 1%	−0.179 (−0.044 to −0.395)
P.m.bairdii	3	Laboratory	16	1600	1600	545	592	> 5%	−0.041 (−0.142 to 0.192)

(9) arrived at the same conclusion in a study of the ability of small mammals to differentiate between sound and empty cocoons of the European spruce sawfly.

The above experiments indicate that the shrews can differentiate between cocoons containing living, healthy sawfly prepupae and cocoons containing mature parasite larvae or parasite pupae. It is of interest to determine whether cocoons containing younger and therefore smaller parasite larvae could still be distinguished from cocoons containing living, healthy sawfly prepupae.

The effect on selection of the size of the parasite larvae was determined from cage experiments conducted under field conditions using one *S.c.cinereus*. This animal was trapped in a sawfly infested plantation and was tested immediately after its capture. The experiments were divided into four consecutive stages. In the first (15 experiments) and third stages (20 experiments) healthy cocoons and cocoons containing mature parasite larvae or parasite pupae were presented. In the second (six experiments) and fourth (six experiments) healthy cocoons and cocoons containing young parasite larvae were presented. In all, 1273 cocoons were opened in the first and third stages and 452 in the second and fourth stages. In all experiments the animal opened more healthy cocoons than parasitized cocoons, corroborating the conclusion that *S.c.cinereus* can differentiate between these two categories. The size of the parasite, however, had an effect on the degree of selection. When cocoons containing young parasite larvae were presented with the healthy cocoons the index of selection was 0.362 (range 0.193 to 0.615). When the larger, mature parasite larvae or parasite pupae were presented the index of selection was 0.565 (range 0.423 to 1.00). That is, the selective ability was significantly greater when the parasites were larger.

The ability of small mammals to differentiate between healthy and parasitized cocoons under the rather unnatural conditions of the cage experiments does not prove that this occurs in the field. Such proof can be obtained from the analysis of cocoons collected from the forest floor. Some of these cocoons are empty and have openings in them. To determine which openings were made by small mammals, caged animals were provided with healthy and parasitized cocoons. The openings made in approximately 4000 cocoons opened by five caged *S.c.cinereus*, 2000 cocoons opened by five caged *B.b.talpoides*, and 5000 cocoons opened by 10 caged *P.m.bairdii* were examined. The jagged, asymmetrical holes chewed in cocoons by small mammals can be distinguished readily from the smooth uniform holes made by emerging sawflies and parasites and from the small holes made by predatory insects such as elaterid larvae. It is also possible to classify the chewed cocoons as to species of small mammal predator (Fig. 2). Cocoons chewed by *S.c.cinereus* invariably have small openings. The holes are elliptical in shape with few abrupt serrations. On the other hand, the openings in cocoons chewed by *B.b.talpoides* and *P.m.bairdii* are larger and more irregular. If the chewed cocoons collected from the forest floor could be segregated into parasitized

and non-parasitized categories, the selective ability of *S.c.cinereus* could be compared to that of *B.b.talpoides* and *P.m.bairdii*. Chewed cocoons, however, can only be identified as parasitized if the parasite has reached the pupal stage. Before pupation the parasite spins a membranous cocoon within the sawfly cocoon and the mammals do not remove this cocoon. The presence or absence of a parasite cocoon within the sawfly cocoon therefore provides a relative index of the number of chewed cocoons that once contained parasites. The selective ability of *S.c.cinereus* thus can be compared to that of *B.b.talpoides* and *P.m.bairdii* on the basis of field data.

The cocoon population in a heavily-infested Scots pine plantation was sampled with 130 sq. ft. quadrats just before adult sawfly emergence in September, 1952. The planatation had been extensively live-trapped during the month of August. *S.c.cinereus*, *B.b.talpoides*, and *P.m.bairdii* were the only mammal predators trapped. Significantly fewer cocoons containing parasite pupae had been opened by *S.c.cinereus* than by *B.b.talpoides* and *P.m. bairdii* (Table III). This indicates that in the field, as in the laboratory, *S.c.cinereus* exhibits a higher positive degree of selection than the other two species. This conclusion assumes that predation by *S.c.cinereus* was proportionate to that by *B.b.talpoides* and *P.m.bairdii* during the time cocoons were in the ground. Extensive trapping from May to September, 1952, in five different plantations indicated that populations of these three species followed the same trend, rising from a low in the spring to a peak in mid-August. The populations of *S.c.cinereus* were proportionate to those of *B.b.talpoides* and *P.m.bairdii* during the time cocoons were in the ground. Presumably the same conclusion could be applied to predation.

Further evidence concerning the selection of healthy vs. parasitized cocoons was obtained from field data. Two samples were collected in the same Scots pine plantation the following year (1953), the first immediately after the sawfly larvae had dropped from the foliage and spun cocoons and the second two and one-half months later. Live-trappings for seven days each month from June to September indicated that *S.c.cinereus*, *B.b.talpoides*, and

TABLE III

COMPARISON OF THE ABILITY OF *S.c.cinereus*, *B.b.talpoides*, AND *P.m.bairdii* TO SELECT NON-PARASITIZED OVER PARASITIZED SAWFLY PREPUPAE

Species	Number of sawfly cocoons opened			% Containing par. cocoon	χ^2
	Lacking par. cocoon	Containing par. cocoon	Total		
S.c.cinereus	1791	185	1976	9.35	64.8**
B.b.talpoides and *P.m.bairdii*	653	168	821	20.5	

** *Difference significant at the 1% level.*

P.m.bairdii were still the common residents. There was a significantly higher per cent parasitism in the sound, unopen cocoons of the second sample (Table IV). This increase could hardly result from parasitism of cocoons since approximately 80% of the parasites were *Exenterus canadensis* and this parasite parasitizes larvae before spinning. Of the remaining parasites, one, *Dahlbominus fuscipennis*, was known to parasitize cocooned larvae. Cocoons parasitized by this insect therefore were not included in compiling data for Table IV. It seems likely that the increase can be attributed to selection of healthy over parasitized cocoons by at least some of the small mammal predators.

The results from cage experiments and from analysis of cocoons collected on the forest floor indicate that small mammals can differentiate between sawfly cocoons containing living prepupae and those containing fungus or parasites. All species avoid opening cocoons containing fungus. The shrews can also differentiate between healthy and parasitized cocoons before opening them. *S.c.cinereus* apparently open more cocoons containing living sawfly prepupae than those containing parasites while the reverse seems to be true of *B.b.talpoides*. *P.m.bairdii* apparently cannot differentiate between the latter two categories. There is a gradation in selective ability that is related to the degree of insectivorous habits of the small mammals. This gradation, in decreasing order of selective ability and of insectivorous habit, is as follows: *S.c.cinereus*, *B.b.talpoides*, and *P.m.bairdii*.

Development of Selective Behavior

While the selective behavior of three species of small mammals has been demonstrated, it remains to trace the development and phases of this behavior. The effect of experience on the selective ability will be considered first. Since the behavior thus far has been described solely in terms of one phase in the search for cocoons, the opening of cocoons, the complex of actions leading to the end response of acceptance or rejection of cocoon contents will also be considered.

Four mature *P.m.bairdii*, born and raised in captivity, were used to determine the effect of experience on the selection of healthy cocoons containing living prepupae and cocoons containing fungus. These animals had been

TABLE IV

A COMPARISON OF THE PER CENT PARASITISM IN PREPREDATION AND POSTPREDATION SAMPLES OF *N. sertifer* COCOONS

Sample	No. healthy cocoons	No. par. cocoons	Total	% Parasitism	χ^2
Prepredation	1200	362	1562	23.17	14.31**
Postpredation	1204	492	1696	29.01	

** *Difference significant at the 1% level.*

isolated from cocoons before the experiments. Each animal was provided daily with 100 cocoons containing living sawfly prepupae and 100 containing prepupae attacked by fungus, in addition to fox chow and water. At the end of each daily experiment the index of selection was calculated on the basis of the cocoons opened. This index represents the average selection from the time the cocoons were provided, at the beginning of an experiment, to the time cocoons were removed, at the end of an experiment. The amount of experience necessary for any index to be attained therefore is taken as one-half the number of cocoons opened in a complete experiment. The changes in the number of cocoons opened and in the indices of selection for one animal, as experience with cocoons was acquired, is shown in Fig. 4. The results for the remaining three animals were similar. The selection curve follows the form of a typical learning curve. After an initial rise the curve reaches a plateau which is fairly constant throughout the remainder of the experiments. The initial rise results from an increase in the number of healthy cocoons opened and a decrease in the number of fungus cocoons opened. The animals thus reacted positively to cocoons containing living prepupae and negatively to cocoons containing fungus.

This learning process is a type of conditioning. The unconditioned or originally effective stimuli are perceived when the cocoons are opened. The conditioned or secondarily effective stimuli are perceived before the cocoons are opened. Just as a dog can be conditioned to salivate in response to a bell (11, 12) so the small mammals are conditioned to open certain cocoons and to avoid opening others, in response to stimuli emanating from the cocoons. Since the index of selection rises as a result of both an increase in the number of healthy cocoons opened and a decrease in the number of fungus cocoons

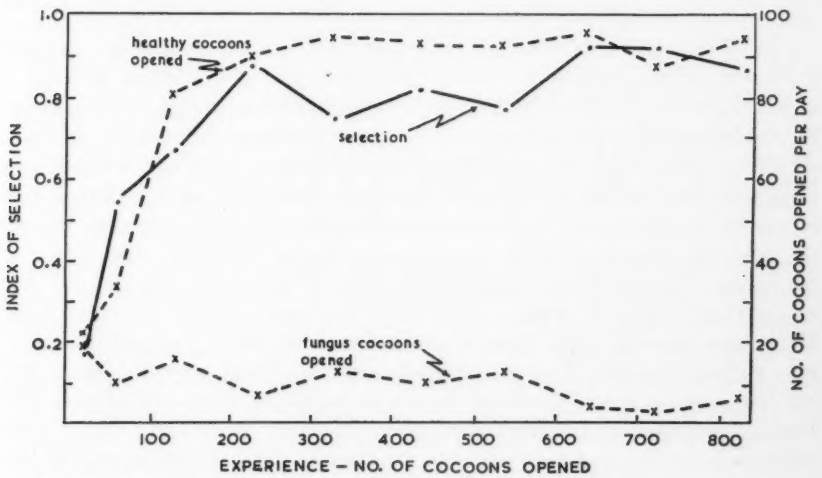

FIG. 4. Effect of experience on the number of healthy and fungus cocoons opened and on the selection between these two types of cocoons by *P.m.bairdii*.

opened, the conditioning involves elements of reward and punishment. The selection curves, one of which is shown in Fig. 4, differ from usual experimental conditioning curves in the number of trials necessary to learn. The number of trials necessary to learn is assumed to be the total number of cocoons opened before the index of selection becomes constant. An average of 157 trials with a range from 57 to 234 was necessary for a plateau to be reached in the experiments with the four *P.m.bairdii*. Normally animals require fewer trials for experimental conditioning; for example, Pavlov (12) showed that from one to 20 trials were necessary to condition the salivary reflex of dogs to the odor of camphor or amyl acetate. The time sequence between the presentation of the conditioned and unconditioned stimuli were rigidly controlled by Pavlov in these experiments. This time sequence is quite variable, however, while an animal is learning to select cocoons, since at times the conditioned stimuli are received and are not immediately reinforced. Since the time sequence between the presentation of the two stimuli has proved to be important in determining the rate of conditioning (10, 11), this variability might be sufficient to account for the relatively large number of trials necessary for learning.

A similar type of learning was observed in the selection of healthy vs. parasitized cocoons by one *S.c.cinereus*. Before the selection or testing period the animal was provided with healthy cocoons (pretesting period). The effect of experience on the index of selection is shown in Fig. 5. As before, there is a gradual rise in the degree of selection with increasing experience. The graph differs, however, from the previous learning curves (Fig. 4) since there was an initial avoidance of the category of cocoons provided in the pretesting period, i.e. the healthy cocoons. This was followed ultimately by an avoidance of the parasitized cocoons.

It is of interest to determine if the selection of healthy vs. fungus cocoons is affected by providing animals with healthy cocoons or fungus cocoons for a period before the actual testing period. To test the effect of such pretreatment 12 *P.m.bairdii* were divided into three groups of four. The animals were chosen from the offspring of four females and were born and raised in captivity. Each group contained one member of each of the four families. Within the groups there were equal numbers of males and females. For one week before the pretesting period, animals were placed in the experimental cage. In the pretesting period each animal of group one was provided with 100 healthy cocoons each night for seven nights, those of group two were provided with 100 fungus cocoons, while those of group three remained in the experimental cage without cocoons. Fox chow biscuits and water were always present. In the following selection, or testing period, each animal was provided with 100 healthy cocoons and 100 fungus cocoons each night for seven nights. Two animals in each group received male cocoons in the pretesting and testing periods, while the remaining two received female cocoons. Experiments were conducted using six animals at one time (two from each group).

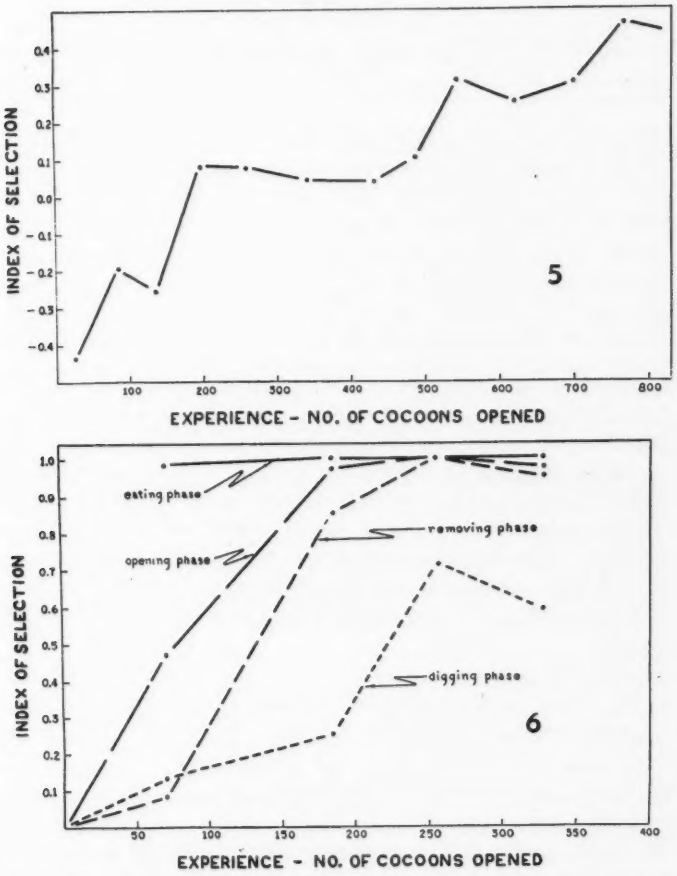

FIG. 5. Effect of experience on the ability of *S.c.cinereus* to select healthy over parasitized sawfly cocoons. The animal was previously conditioned to healthy cocoons.

FIG. 6. Effect of experience on the selective ability (healthy vs. fungus) of one *S.c.cinereus* in different phases in the search for cocoons. The animal had been isolated from cocoons for five months before the experiments.

When the index of selection was plotted against the cumulative number of cocoons opened, the graphs were similar to those in Fig. 4. There was invariably an initial rise in each graph followed by a plateau. Pretreatment, however, apparently affected the height of the plateau. The number of cocoons of each category opened after the curves levelled are listed in Table V. The indices of selection differ between any two types of pretreatment. The selective ability decreases in the following order: animals pretreated to healthy cocoons, those not pretreated, and those pretreated to fungus cocoons. Since insufficient cocoons were available to extend the selection period beyond seven nights, it is not known how long these differences would be maintained.

TABLE V

EFFECT OF PRETREATMENT ON THE SELECTIVE ABILITY (HEALTHY VS. FUNGUS) OF 12 *P.m.bairdii*

Cocoons used in pretreatment	No. expts.	No. cocoons provided		No. cocoons opened		Index of selection
		Healthy	Fungus	Healthy	Fungus	
Healthy	18	1800	1800	1466	109	0.864
None	16	1600	1600	978	99	0.816
Fungus	19	1900	1900	1654	231	0.755

As a further test the pretreatment of two animals used in the above experiments was changed after a three to five month isolation from cocoons. One animal (160*B*), previously provided with fungus cocoons in the pretesting period, was provided with equal numbers of both categories without pretreatment to either category. The other (257*D*), previously provided with healthy cocoons in the pretesting period, was provided with fungus cocoons before the selection period. The results are presented in Table VI. With each animal there was a highly significant change in the index of selection when the pretreatment was changed. Also, the range of the indices between experiments did not overlap.

The criterion for selection up to this point has been described mainly in terms of one phase in the search for cocoons. This phase, the opening of cocoons, is only one of a series which culminate in the eating of the contents

TABLE VI

EFFECT OF A CHANGE IN PRETREATMENT ON THE SELECTIVE ABILITY (HEALTHY VS. FUNGUS) OF TWO *P.m.bairdii*

Pre-treat-ment	Animal	Date	No. cocoons provided		No. cocoons opened		Av. index of selection and range	χ^2
			Healthy	Fungus	Healthy	Fungus		
Fungus	160B	Dec. 12 −18	600	600	515	43	0.846 (0.794-0.916)	21.3**
None		May 5 −11	600	600	571	11	0.962 (0.938-0.981)	
Healthy	257D	Dec. 8 −13	500	500	433	18	0.920 (0.865-0.977)	7.14**
Fungus		Mar. 10 −15	400	400	386	35	0.833 (0.822-0.834)	

** *Difference significant at the 1% level.*

of cocoons. For a complete analysis of the behavior all the phases should be considered. Selection in four of these phases was studied. These included a digging phase, a removing phase, an opening phase, and a final, eating, phase when the cocoon contents were eaten.

Information on the characteristics of selective behavior in these phases was obtained by studying the ability of *S.c.cinereus* to differentiate between healthy cocoons containing living prepupae and cocoons containing fungus. The experimental method was similar to that used in other cage experiments except that the positions of healthy and fungus cocoons were known. Each day 150 cocoons of each category were placed alternately in a grid pattern on the floor of a cage measuring 30 × 24 × 6 in. Fine washed quartz sand was then carefully sprinkled over the cocoons to a depth of two centimeters. This was dampened and pressed so that the holes dug by the animal would remain clear. At the end of each experiment the sand was sifted and the cocoons removed. Fig. 3 shows the appearance of the experimental cage after a *S.c.cinereus* had been in it for one night. Cocoons were buried where the lines of the removable grid intersect. Since, as the photograph shows, the holes were dug directly over cocoons, selection in the first or digging phase could be measured. The criterion for selection in the removing phase was obtained by counting the number of cocoons of each category removed from the soil, in the opening phase by counting the number opened, and in the eating phase by counting the number from which the contents were removed.

Experiments were conducted with three *S.c.cinereus*. Fig. 6 presents the results of a typical experiment with one *S.c.cinereus* isolated from cocoons for five months before the experiments. Cocoons containing living sawfly prepupae were selected in each phase. In all phases except the final, eating, phase, the selective ability markedly increased as experience was gained. The selective ability varied between phases when the learning was complete. The selective ability in any given phase was higher and more precise the closer that phase was to the final, consummatory act. The final phase did not, in these experiments, show any variation with experience. It, apparently, is "rigidly" determined.

The external stimuli releasing and directing the behavior in the various phases might account for the gradation in precision of selection. The effective stimuli may be supplemented by others as the animal passes from one phase to another. In the digging phase, the animal is exposed solely to nonvisual and non-tactile stimuli while in the removing and opening phases, visual and tactile stimuli can be perceived. In the final phase, gustatory stimuli are added to the complex. The gradation in degree and precision of selective behavior may therefore result from an addition of stimuli. There should be no differences in the selective ability in the removing and opening phases if this is the only explanation because in these phases the same stimuli must be involved in selection. Since there is a difference, the gradation is probably a characteristic of the behavior itself and not of the external factors releasing and directing it.

Discussion

As was noted in the introduction this problem arose from a study of the effect of small mammal predation in controlling populations of the sawfly *N. sertifer*. The results presented in this paper can be interpreted in terms of control. Small mammal predators avoid sawfly prepupae attacked by other control factors such as fungus and parasites. The interference of small mammal predation on parasitism and disease is particularly reduced in the highly insectivorous mammals. Since parasites are avoided, these are left to reparasitize the subsequent generation of sawfly larvae.

This is only part of the problem. Predation, and parasitism and disease, may be mutually interfering. For example, the presence of cocoons containing fungus conceivably could affect the predation. That is, the violent avoidance of fungus cocoons might extend to any cocoon with the same visual and tactile appearance. Predation on living sawfly prepupae therefore would be reduced.

Because of this mutual interference between control factors, their control is difficult to assess. A mere knowledge of the percentage of cocoons opened or parasitized is insufficient. An example will clarify this statement. Let us presume that in a sample of 100 cocoons, 25 contained parasites, 25 contained prepupae attacked by fungus, 25 were opened by small mammals, and 25 contained living sawfly prepupae. The control by small mammals would appear to be 25%. If the open cocoons were opened by a mammal that completely avoided parasites and prepupae attacked by fungus, then the per cent control of the sawfly cocoons would actually be 50%. The calculated per cent control by parasites in turn would drop from 50% to 33%. The problem is made even more complex by the presence of different species of small mammals with different selective abilities. Further complexities are added by the gradual increase in the degree of selection concomitant with the increase in size of the parasite larvae. These complexities can be partially eliminated by an exhaustive study of the feeding behavior of individuals of all species of small mammal predators.

Acknowledgments

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THE FECUNDITY OF *AEDES HEXODONTUS* DYAR (CULICIDAE) IN THE LABORATORY[1]

By C. A. Barlow[2]

Abstract

The numbers of eggs laid by 654 *Aedes hexodontus* females, captured in the field, were examined under laboratory conditions in relation to three variables: nutrition, weight, and date of capture of the individuals. Females were weighed before and after engorging with blood and maintained in individual cages at a constant temperature of 20° C. The mean fecundity of 198 females was 62.3 ± 2.16 eggs. A blood meal was necessary for oviposition in 95% of the individuals. Five per cent of the females oviposited without a blood meal. A diet of a single blood meal and water was insufficient for oviposition and either sugar or raisins was required to supplement the single blood meal. There was a definite correlation between weight and fecundity. Vertebrate blood was a stimulus to egg development. A critical minimum amount of blood was required to initiate ovulation, but blood in excess of this did not increase the number of eggs. No correlation was shown between the fecundity of females in the laboratory and the date of their capture.

Introduction

The population of mosquitoes around Churchill, Manitoba, during the summer, has been estimated to be five million individuals per acre. Much of this population is composed of individuals of the species *Aedes hexodontus* Dyar. This mosquito breeds typically in open tundra pools. The adults emerge in late June or early July and persist until the latter part of August. *A. hexodontus* is a univoltine species with an obligate diapause in the egg stage.

The present study was undertaken to determine the number of eggs laid by *A. hexodontus* females, and the effects upon this characteristic of nutrition, weight, and date of capture of the adult females. Because the adults will not mate in small cages, and because of the obligate diapause which occurs in the egg stage, all individuals used in the experiment had to be captured in the field.

Most of the previous work relating to the fecundity of mosquitoes has referred to the influence of vertebrate blood on the number of eggs laid. An extensive bibliography on the subject is given by Bates (1).

Materials and Methods

A. hexodontus females were collected at intervals throughout the summer from a typical area of forest transition near Churchill, Manitoba. These individuals were transported in cotton stoppered shell vials to the laboratory where they were identified. The shell vials and their cotton wool stoppers were thoroughly dry; this prevented the females from obtaining any liquid just before they were weighed. Care was taken to choose females for the experiment which had not had a previous blood meal, as far as observations of the abdomens could determine.

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Contribution from the University of Toronto, Toronto, Ontario. Based on a thesis submitted in conformity with the requirements for the degree of Master of Arts, University of Toronto, 1954.
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Females were weighed twice, once before obtaining a blood meal and again immediately after, so that the weight of blood consumed could be determined. Each individual was weighed to the nearest 0.01 mgm.

Carbon dioxide was used to subdue the mosquitoes during weighing. Recovery of the females from slight CO_2 asphyxiation was rapid and seemed to leave no after effects. Chloroform and ether were also tried. The former, even in small doses, nearly always killed the individual; recovery from ether was slow and left the insect lethargic.

Immediately after the first weighing, each female was allowed to engorge from a guinea pig and then was weighed a second time. Attempts were made at first to regulate the extent of engorgement of individuals by disturbing them as they were feeding. This treatment was abandoned because once the female had begun to draw blood, it would not withdraw its proboscis unless treated roughly. Such treatment often broke the stylets of the mosquito within the guinea pig and resulted ultimately in the death of the female.

After the second weighing each female was placed in a lantern globe oviposition chamber, previously described by Beckel (2). The top of each globe was covered with a piece of cheesecloth over which was placed a cardboard plaque. According to which accessory diet was supplied to the insect, each plaque was equipped with either three moist raisins on a pin, or a small length of glass tubing containing a piece of cotton wool soaked with 15% solution of table sucrose and projecting from the lower end. The pin with raisins or the tube with its sugar soaked cotton was suspended inside the globe by insertion through a small hole in the cheesecloth cover. This arrangement allowed easy access to the food but eliminated the possibility of the female becoming entangled as it might have if the food had been placed on the bottom of the cage.

The globe was set on a Petri dish filled with wet cotton wool. The cotton was covered with filter paper and served as a site for oviposition and as a supply of water. Distilled water was added each day to the Petri dishes. Separating the filter paper and the lantern globe was a square of plastic screening placed directly on the filter paper. The mesh of the screen was small enough to retain the mosquito but large enough to allow it to oviposit through the screen onto the damp filter paper.

Every two days the raisins were changed and fresh sugar solution added from a pipette through the top of the glass tube. The cheesecloth which covered the globe simplified the changing of the raisins since the plaque containing the pin and raisins could be removed and fresh raisins added while the small hole in the cloth was plugged with cotton wool. Raisins were soaked overnight in distilled water before they were used.

Room temperature during the experiment was controlled at 20° C. Relative humidity in the room varied between 6% and 83% throughout the experiment. The humidity inside each lantern globe was probably relatively constant and higher than in the room itself since the globes were set over the wet oviposition sites and were closed partially at the top.

Individual records were kept of the time oviposition began, the number of eggs laid, and when each insect died. To avoid disturbing the mosquitoes, eggs were not removed and counted until the ovipositing females had died.

Each day that blood-fed females were added to the experiment, a comparable number of females were also set up as controls. The latter received the same foods as the blood-fed females, i.e. either sugar or raisins, but were not given a blood meal. A total of 654 females were used. Depending on the diet supplied to each, these females were divided into the following five categories: controls fed 15% solution of table sucrose, controls fed raisins, single blood meal and water, single blood meal and 15% solution of table sucrose, single blood meal and raisins. The number of insects in each group is shown in Table I.

TABLE I

RELATION BETWEEN FOOD AND NUMBER OF EGGS LAID

Diet	Total No. of individuals	No. of females productive	Total No. of eggs laid	Mean fecundity of productive females
15% solution of table sucrose	108	6	32	5.3
Raisins	102	5	34	6.8
Single blood meal and water	116	None	—	—
Single blood meal and 15% solution of table sucrose	163	106	6272	59.17 ± 2.91
Single blood meal and raisins	165	103	6691	64.96 ± 3.00

Results

Influence of Nutrition

Necessity for Vertebrate Blood

As Table I indicates, only those females which received a blood meal oviposited, with the exception of 11 individuals of the experimental controls, i.e.: fed either sugar or raisins but no blood. None of the latter specimens laid more than 11 eggs. These 11 females which laid eggs without a blood meal in the laboratory could have acquired, previous to capture, a food which was sufficient to mature a few eggs. However, the time required for ovulation in each of these females was within the range observed for females which received a blood meal in the laboratory. Probably then, the few eggs laid were matured on diets of sugar or raisins provided in the laboratory and were not the last of batches of eggs developed on blood meals obtained in the field.

Effect of a Single Blood Meal Only

No eggs were laid by the 116 females which were given a single blood meal and water only (Table I). Dissections of individuals of this category showed

that the eggs developed to a certain stage but no oviposition occurred. This observation suggests that starvation was the reason for the lack of oviposition.

The utilization of food energy by these insects is considered to have been the result of the simultaneous operation of two physiological systems. One served to maintain the life of the organism while the other system was responsible for the development of reproductive products. But when the amount of food available was insufficient to maintain egg development as well as the basic life processes of the insect, ovulation nevertheless continued, and hastened the death of the individual by starvation.

Effect of a Single Blood Meal with an Accessory Food

With the exception of the previously mentioned 11 females, eggs were laid only by females which received a constant supply of either sugar solution or raisins in addition to a single blood meal. Table I shows that a mean of 59.17 ± 2.91 eggs were laid by 106 individuals which received a single blood meal and sugar and a mean of 64.96 ± 3.00 eggs were laid by females which were fed a single blood meal and raisins. These two means are not significantly different. The mean number of eggs laid by 198 females from both categories was 62.25 ± 2.16.

Relation Between Fecundity, Weight of Female, and Weight of Blood Meal

Fig. 1 shows that fecundity was correlated with weight of female. A correlation between fecundity and weight of blood meal was also indicated (Fig. 2). Thus fecundity seems to have been correlated with both weight of

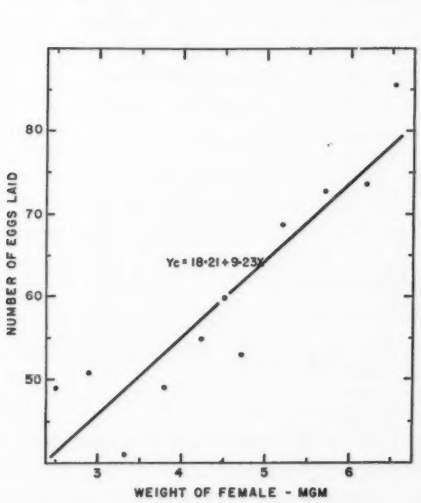

FIG. 1. Relation between fecundity and weight of female. Each point represents the mean of a number of females.

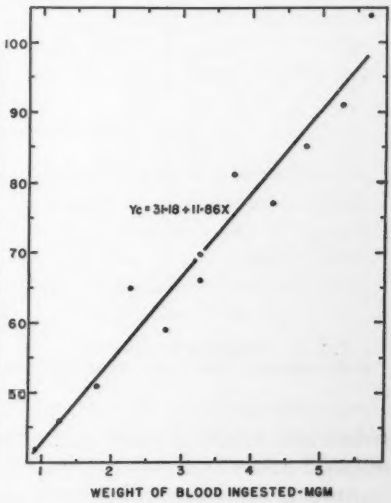

FIG. 2. Relation between fecundity and weight of blood ingested. Each point represents the mean of a number of females.

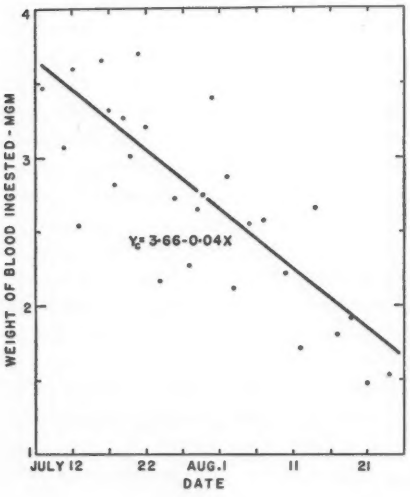

FIG. 3. Relation between weight of blood ingested and date. Each point represents the mean of a number of females captured and fed on that particular date.

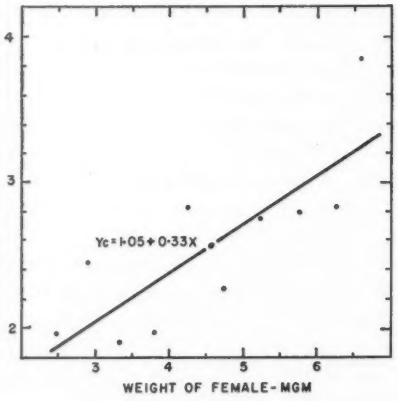

FIG. 4. Relation between weight of blood ingested and weight of female. Each point represents a mean value.

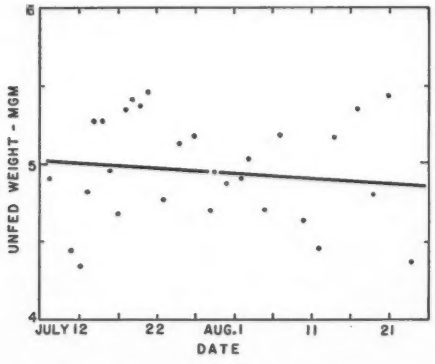

FIG. 5. Relation between weights of females captured in the field and the dates of their capture. Each point represents a mean value.

female and weight of blood meal. However, Fig. 3 illustrates that the weight of blood taken by females in the laboratory decreased with the date of their capture and feeding throughout the experiment. But neither weight of female on the date of capture (Fig. 5) nor fecundity decreased over the same period of time. Thus Figs. 1, 3, and 5 show that fecundity was dependent on weight of female rather than on weight of blood consumed.

Serial sections of females used in the experiment also showed that fecundity was directly related to weight of individual. Females which received only a single blood meal and water did not oviposit but eggs matured in all existing ovarian follicles. It is considered that the food available to any female was used both for egg development and for maintenance of life of the female. A single blood meal was insufficient to support both these processes. It would not be expected, then, that females which received only this single blood meal would produce a mass of eggs comparable to that produced by females which received sufficient food for oviposition, if fecundity depended on the weight of blood available. It may be argued that a small amount of blood could have been sufficient for development of only a portion of the follicles in the ovary. But that phenomenon was not observed. If ovulation occurred at all, eggs developed in all existing follicles.

In addition the data indicate that a critical minimum weight of blood was required to initiate egg development. Females which received less than 0.51 mgm. of blood did not oviposit. There might have been a range of critical weights of blood depending on the sizes of the individuals.

Then fecundity was influenced mainly by the weight of the female as long as a certain minimum requirement of blood was consumed. The correlation between fecundity and weight of blood meal probably resulted because larger females tended to consume more blood than smaller individuals (Fig. 4).

In Figs. 1 and 4 the spread of points about the calculated regression lines is greater than in Fig. 2. That is, functions relating to the weights of females (Figs. 1 and 4) were not as clearly expressed as the relation involving weight of blood ingested (Fig. 2). This difference might have occurred because the "basic" weights of the females were not obtained. The individuals undoubtedly consumed moisture of some kind before capture. A better criterion would have been the size of the ovaries which was actually what the weights of the females were meant to represent.

Influence of Date of Capture

Previous observations suggested that females which were captured later in the period of activity of the species laid fewer eggs than individuals which were captured earlier. No significant change in fecundity with date of capture of the females was indicated by this experiment. Also, as previously stated, weight of the female, the characteristic considered directly correlated with fecundity, did not change significantly with date of capture (Fig. 5).

Discussion and Conclusions

Differences between the fecundities of different species of mosquitoes have been emphasized by various authors (Roy (10), Marshall (7), Bates (1)) and imply that fecundity is genetically determined. The present work has shown that fecundity was dependent also on food, a factor of the external environment of the mosquito. Then the observed fecundity of any *A. hexodontus* individual is considered to have been the result of environmental influence on the genetically determined potential fecundity of that individual.

The observation that 11 females laid eggs without a blood meal in the laboratory indicates a tendency for egg development without blood among individuals of the species. However, this characteristic must not be very widespread in the species as it occurred in only five per cent of the specimens.

A number of cases of oviposition without a blood meal have been described in the literature (Roubaud (9), Fielding (3), Marshall and Staley (8), Woodhill (13)) for species which normally take blood. Roubaud proposed the term "autogeny" to signify this characteristic in *Culex pipiens* L. Among most species of mosquitoes, however, egg development seems to depend on the consumption of a blood meal.

The inadequacy for *A. hexodontus* of a single blood meal, if it also exists under natural conditions, is no problem there. Several statements have been published (Theobald (12), Knab (6), Hocking *et al.* (5)) of observations of culicines feeding on nectar. This adaptation to a different type of food in the field is necessary to maintain a female before it finds a blood meal and during the development of eggs.

In this experiment, sugar solution served as an accessory food. Hecht (4) obtained similar results with *Anopheles maculipennis* Meigen and stated that the effect on egg development of a sugar diet was one of "stimulation". However, in the present experiment ovulation occurred in females which had had only a single blood meal. That oviposition did not take place was not because of lack of stimulation and such a characteristic cannot be attributed to the accessory diet in this case. Rather it sustained the insect until it had matured and laid its eggs.

Previous literature has largely neglected weight of a female mosquito as a possible influence on its fecundity. One consideration of this factor is by Roy (10) who concluded that "the determining factor in the numerical productivity of eggs is by no means the weight of the mosquito but probably depends entirely on the weight of blood feed". Roy offered two explanations for the influence of blood on ovulation: (1) a physicochemical or qualitative stimulus to the ovarian follicles, or (2) some constituents in the blood may furnish nutrient material, i.e.: the role of blood in ovulation may be quantitative.

It is difficult to understand how ingested vertebrate blood could increase the number of follicles in the ovary of the adult mosquito. This would have to be the case if fecundity depended solely on the weight of blood digested by the female, since egg development appears to be an "all or none" process.

From the results of the present experiment it is concluded that the observed fecundity was dependent upon size of female, upon a stimulus provided by vertebrate blood, and perhaps upon other environmental factors. The fecundity of any individual was limited by weight of blood meal only below the amount required to stimulate ovulation in that individual. After egg development was initiated, food other than blood was sufficient to complete the process.

No correlation was shown between the fecundity of *A. hexodontus* females and the date of their capture. Such a change in fecundity during the season

could be caused by some physiological alteration in the female. In species which hibernate as adults, egg development and oviposition are usually subject to seasonal variation. Prehibernation blood meals may serve for the development of a large reserve of fat instead of for the development of eggs. This state in *Anopheles atroparvus* van Thiel was termed "gonotrophic dissociation" by Swellengrebel (11). A similar condition does not occur in *A. hexodontus*, however, as it is a univoltine species with diapause in the egg stage. The female is reproductively active throughout its lifetime.

Acknowledgments

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THE EFFECTS OF THYROXINE AND GONADAL STEROIDS ON THE ACTIVITY OF SALMON AND GOLDFISH[1]

By W. S. Hoar,[2] M. H. A. Keenleyside,[3] and R. G. Goodall[3]

Abstract

Goldfish immersed in a solution of thyroxine, testosterone, or stilboestrol are more active than untreated fish. The strength of electrical stimulus required to produce a standard response is also less in similarly treated goldfish. Coho and sockeye salmon yearlings tested with the same hormones and sockeye salmon yearlings treated with dienoestrol or nylestin show a decrease in time required to make a standard response in flowing water. These data are discussed in relation to the appetitive behavior of migrating fishes.

Introduction

The stimulating effects of thyroid hormone and the gonadal steroids on the locomotor activity of a wide variety of vertebrates is recognized (3). Elsewhere it has been argued that these hormones, by increasing the responsiveness of fish to stimuli, may play an important part in sensitizing the organism to directive factors involved in migration and in stimulating the locomotor activity necessary for the prolonged journeys undertaken by many fishes (7, 9). In this way the hormones are probably involved in appetitive behavior although they may also be associated with consummatory acts (14).

There are relatively few experimental demonstrations of the effects of hormones on the general activity and responsiveness of fish. Fontaine (5) reported that elvers of *Anguilla anguilla* showed a marked decrease in rheotactic behavior when treated with the antithyroid compound, phenylthiourea (1/10000) and suggested that thyroid hormone stimulates the strong positive rheotaxis displayed by these animals when they are entering rivers. Stanley and Tescher (13) recorded a 400% increase in the locomotor activity of goldfish (*Carassius auratus*) fed on beef testes and attributed this effect to the gonadal hormones in the diet. Hoar, MacKinnon, and Redlich (10) treated coho (*Oncorhynchus kisutch*) and chum (*O. keta*) salmon fry with synthetic thyroxine, methyl testosterone, or thiourea and observed changes in the rate of swimming and in the intensity of schooling among the chum salmon and in the aggregating tendencies of the coho.

Many experiments have been designed to study the effects of reproductive hormones on the behavior of reproduction (9). However, the three referred to above were carried out to test more specifically the effects of hormones on general activity, and the results support the idea that thyroxine and the gonadal steroids do stimulate general activity in fishes. The present

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investigation was designed to provide additional experimental evidence and to test more particularly the effects of hormones on the intensity of the response made to a variety of stimuli.

Materials and Methods

Goldfish, *Carassius auratus*, were obtained from the Goldfish Supply Company, Stouffville, Ontario, and ranged in length from 6 to 9 cm. They were maintained under standard aquarial conditions at 20° ± 2° C. and fed twice daily on pablum and a commercial fish food consisting largely of shrimp and fish meal.

Yearling salmon were collected from streams in the lower mainland of British Columbia and cultured in running water for several months prior to the experiment. They were fed a mixed diet of pablum, canned salmon, fish meal, and beef liver. The mean fork length for 20 coho was 8.9 (standard deviation, 0.54 cm.) and for the same number of sockeye 8.2 (S = 0.53 cm.).

Hormone Treatment

Fish were treated by immersion in solutions of the hormones. Groups of 20 goldfish were held at room temperature (20° ± 2° C.) in 36-liter glass aquaria containing 28 liters of solution. Salmon were treated in groups of the same size maintained in 22-liter battery jars containing 20 liters of solution. The battery jars were placed in running water and all experiments on salmon carried out at the temperature of tap water (6° to 9° C.). Fish were fed twice daily and solutions changed three times per week.

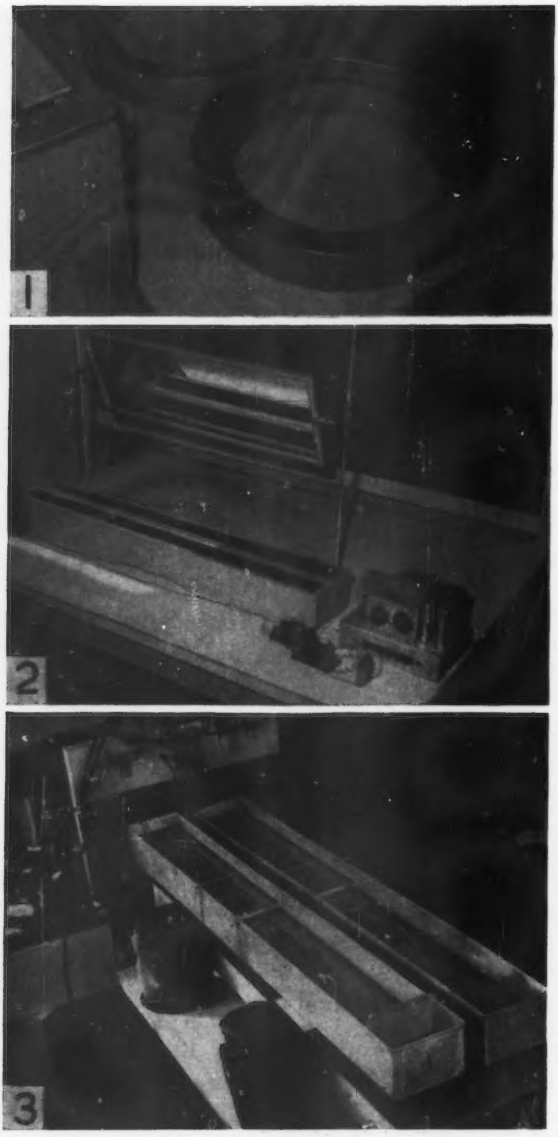
In the case of thyroxine and testosterone the concentrations of immersion solutions were based on previous experiments with salmon and goldfish. Concentrations of the synthetic estrogens were dictated by availability and some potency data supplied by British Drug Houses. All drugs were purchased from British Drug Houses and used as follows:

Synthetic thyroxine sodium	1 : 2,500,000
Methyl testosterone (testaform)	1 : 2,000,000 for salmon and 1 : 2,800,000 for goldfish
Stilboestrol	1 : 1,600,000 for salmon and 1 : 2,500,000 for goldfish
Dienoestrol	1 : 1,600,000
Ethinyl oestradiol (nylestin)	1 : 20,000,000

Apparatus

(*a*) *Amount of Swimming by Goldfish*

The amount of swimming by goldfish was observed in a pair of circular channels with an outside diameter of 90 cm. and with channels 10 cm. wide and 15 cm. deep (Fig. 1). The circumferential distance at the center of the channels was 251.3 cm. Water was about 8 cm. deep. The channels were constructed of galvanized iron and painted with aluminum paint. The



Apparatus used to compare reactions of hormone treated fish.

FIG. 1. Circular channels used to measure amount of swimming.

FIG. 2. Wooden troughs (120 cm. long, 5 cm. wide, and 7.5 cm. deep, inside dimensions) with viewing mirror and stimulating electrodes.

FIG. 3. Troughs used in jumping experiments to show pump and technique for circulating and cooling water.

bottom of each channel was marked with eight equally spaced transverse black lines. Activity was measured by recording the total number of lines crossed by three fish in each of the two channels during three 10-min. periods (one in the morning, at about 9 a.m., one at noon — 12.30 to 1.30 p.m. — and the third in the late afternoon at about 5 p.m.). In this way adjustments were made for diurnal variations in activity. Fish were placed in the channels during the evening of the day previous to observation.

(*b*) *Responses to Electrical Stimulus*

Responses of goldfish to electrical stimulation were measured in wooden troughs, similar to those used by Elson (4). Four troughs, in pairs with an overhanging mirror for observation (Fig. 2), could be observed at the same time. A suitable series of switches permitted discharge of the microfarad condenser through any desired trough. The voltages used were readily recorded from the voltmeter on the rectifier as illustrated in Fig. 2. The sterling silver electrodes were 5 cm. by 7.5 cm. and 0.1 cm. thick. Single fish were placed in the troughs at least eight hours prior to study. Commencing with a subthreshold voltage, stimulation was carried out at one minute intervals with increasing voltages until the fish darted 15 cm. when stimulated. This voltage was termed the "maximum response voltage". Occasionally the fish did not dart 15 cm. even under maximal stimulation. In this case the voltage which produced the greatest response was the "maximum response voltage". As in the case of the activity experiments, the procedure was carried out three times per day to eliminate diurnal fluctuations in activity. Individual goldfish were used only once in these tests.

(*c*) *Stimulation by Water Currents*

The jumping behavior of salmon yearlings was studied in a pair of galvanized iron (aluminum painted) troughs 225 cm. long. These have been previously described (8) and are illustrated in Fig. 3. A vertical dam divides each trough into two equal areas. The difference between the water levels of the upper and lower pools was 2.0 cm. for the coho and 1.5 cm. for the sockeye. The water volume entering each trough was 14 liters per min. and was recirculated as shown in Fig. 2 by a pump and a catch basin with a refrigerated reservoir at the inflow end. Temperatures were maintained at those of the running water where the fish were held between experiments.

In these experiments 20 fish were removed from the immersion baths to a pail of fresh water and held for one hour while flows and temperatures were being adjusted in the apparatus. They were then poured into the lower pool and observations were begun at once from behind a screen. The time required for 50% of the sample (i.e., 10 fish) to jump from the lower to the upper pool was recorded as the reaction time. Fish were immediately returned to the aquarium. Groups were tested from three to four times (Tables II and III) during each of the second, third, and fourth weeks of treatment and the results averaged for each week. Experiments were repeated with the thyroxine

and testosterone treated salmon but were performed only once with the estrogens. Thus, the number of tests in one week is as high as eight or nine (Table II) for the testosterone and thyroxine treatments but never more than six for the estrogens (Table III).

Results

Activity of Goldfish in Circular Channels

In the first experiment the control test (duplicate channels) was carried out prior to treatment. Fish were then tested after 5, 10, and 15 days of immersion (Fig. 4). With one exception (10 days of treatment with thyroxine) activity was considerably greater after hormone treatment. Of all the fish tested those immersed for 10 days in testosterone showed the greatest effect. Thyroxine and stilboestrol produced their greatest effects during the last period of treatment.

In the second experiment a separate control group was maintained and tested at the various intervals. Results with the experimental fish were compared with controls studied during the same period. The data (Table I) are presented to show diurnal variation as well as the changes caused by hormone immersion.

On the whole hormone treatment produced marked increases in the amount of swimming activity. In this experiment the effect with thyroxine and with stilboestrol is greatest in the last test while testosterone reaches an early peak and declines. These differences may be related to the response of the tissues or to the dosages used. A diurnal rhythm in activity is evident. The fish are, on the whole, two or three times more active in the late afternoon than they

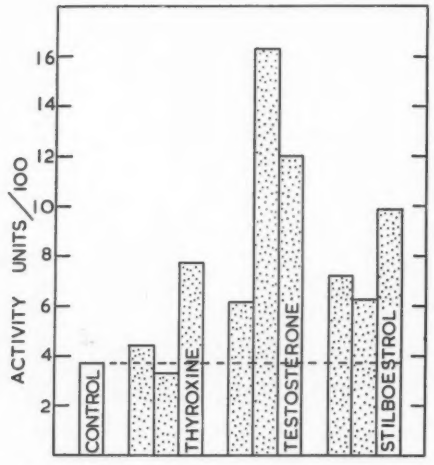


FIG. 4. Activity of goldfish. Total number of lines crossed in circular channels during three 10-min. periods (a.m., noon, and p.m.) recorded with three fish in each of two channels. Three bars in the experimental groups from left to right show values for 5, 10, and 15 days' hormone treatment.

TABLE I

ACTIVITY OF GOLDFISH

Activity units are numbers of lines crossed in duplicate channels each containing three goldfish. Values recorded are deviations from control value obtained with untreated goldfish maintained for the same period under conditions otherwise comparable to the experimental groups. Positive values unless otherwise shown.

Treatment	Time of day	Activity units after immersion for 5–8 days	11–14 days	18–21 days	Total units
Thyroxine	A.M.	100	124	190	414
	Noon	36	−34	176	178
	P.M.	350	380	246	976
	Total, units	486	470	612	
Testosterone	A.M.	160	84	50	294
	Noon	250	104	64	418
	P.M.	336	150	246	732
	Total, units	746	338	360	
Stilboestrol	A.M.	−36	20	330	314
	Noon	−24	−64	76	−12
	P.M.	170	390	300	860
	Total, units	110	346	706	

are in the morning. The total amount of activity is least at midday although testosterone treated fish were consistently less active during the morning test. It would be interesting to know whether this difference with testosterone has any significance.

Response of Goldfish to Electrical Stimulation

A considerable amount of variability is expected with this experimental procedure (4). For this reason, conditions in the different groups were maintained as similar as possible. The four groups (control and the three hormone treatments) were tested in identical troughs at the same time, thus eliminating variability which might otherwise have arisen from small differences in temperature or time of day.

The findings (Fig. 5) are in line with those just described for amount of swimming in the circular channels. After the first few days of treatment the excitability of the fish rises so that they make the standard response at much lower voltages than do the untreated fish. After extended treatment the effect is less marked.

The high values recorded during the first days of treatment are no doubt due to initial physiological adjustments required in hormone solutions. General observations confirm this suggestion. Occasionally a goldfish (stilboestrol, for example) will die during the first days of treatment and on the whole the fish are less alert and feed less readily than they do after four or

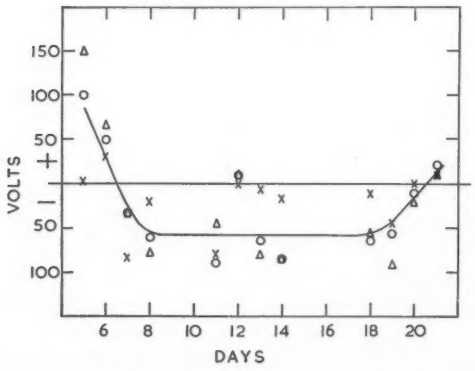


FIG. 5. Response of goldfish to electrical stimulation. Plotted values are voltages in terms of the deviations from the voltage required to stimulate the control fish; average of morning, noon, and afternoon value for the days indicated; circles, thyroxine; triangles, stilboestrol; crosses, testosterone; trend line smoothed through the averages for all three treatments.

five days of immersion. Prolonged treatment may be detrimental and account for the terminal decline in responsiveness. The dosages may have been far greater than physiological amounts occurring normally in fish tissues. From one week to almost three weeks, however, the fish are regularly more easily stimulated than controls. During this period they are alert, always ready to feed and show normal reactions in the aquaria where they are held. Again it is noted that testosterone treated fish reach a peak response earlier and this declines more rapidly than fish treated with the other drugs. However, the over-all effect is so similar with the three treatments that a trend line has been drawn through all the values in Fig. 5.

Response of Salmon to Water Current

The underyearling salmon when introduced into the downstream pool (Fig. 3) show characteristic behavior. During the first few minutes after introduction the fish swim back and forth in close groups from the face of the dam to the outlet screen. Sockeye aggregates are noticeably more compact. Swimming is at first rapid and regularly timed and the fish remain for only a few seconds below the dam. The time spent struggling in the turbulent water below the dam increases markedly in the next few minutes, with the sockeye remaining closely schooled while the coho groups often break up. There was a tendency for the sockeye to remain in the greatest turbulence while the coho sometimes rested near the bottom in front of the dam or swam about in the pool (See also (8)).

Another interesting feature was the tendency for several fish to jump over the dam together. It was repeatedly noticed that several minutes would pass with no jumping and then five or six fish would move over the barrier in a few seconds. This was more evident with sockeye, perhaps because of their schooling behavior (8).

Coho showed two activities never displayed by the sockeye. In 26 coho experiments — 15 with treated fish and 11 with controls — some fish moved back from the upper to the lower pool. The other peculiarity of the coho was its "chafing" behavior. In a chafing movement the fish turns on its side and in darting forward, strikes the bottom with its flank. This behavior is well known to aquarists and has been described in cichlids (1). It has also been noted in this laboratory with the cyprinid *Mylocheilus caurinus* and young kamloops trout (*Salmo gairdnerii*). Chafing occurred both above and below the dam, among control and treated coho, and by fish either facing or moving with the current. Neither downstream movement nor chafing behavior was seen among sockeye.

Quantitatively pretreatment of the young salmon with thyroxine or gonadal steroids reduced the time required for 50% of the sample to reach the upper pool. In detail the data (Tables II and III) show several irregularities and

TABLE II

TREATMENT OF SALMON WITH METHYL TESTOSTERONE AND THYROXINE

Time required for 50% of a sample of 20 treated salmon to jump from the lower to the upper pool. Values given as deviations from control value with t and P values for each treatment with its own control.

	Sockeye					Coho				
			Treated					Treated		
Weeks treated	Tests	Control, min.	Min.	t	P	Tests	Control, min.	Min.	t	P
Testosterone										
2	6	13.8	+8.0	1.951	0.08	4	24.8	−7.5	3.000	0.02
3	9	12.1	−1.3	0.929	0.37	9	26.9	−9.4	3.357	<0.01
4	7	10.5	−2.6	1.857	0.09	9	18.1	−3.2	1.067	0.3
Thyroxine										
2	5	21.9	−3.2	0.561	>0.5	4	23.3	−1.4	0.179	>0.5
3	8	14.2	−3.3	1.100	0.3	8	25.6	−3.2	0.941	0.36
4	8	11.1	−1.2	0.857	0.4	8	18.7	+7.7	2.406	0.03

TABLE III

TREATMENT OF SOCKEYE SALMON WITH ESTROGENS

Time required for 50% of a sample of 20 smolts to jump from the lower to the upper pool. Values given as deviations from control with t and P values for each treatment with the same control.

			Treatment								
			Stilboestrol			Dienoestrol			Nylestrin		
Weeks treated	Tests	Control, min.	Min.	t	P	Min.	t	P	Min.	t	P
2	3	15.8	−5.5	2.037	0.1	+4.1	0.569	0.5	+4.5	0.803	0.46
3	6	8.2	−0.7	0.854	0.4	+3.7	3.083	0.01	+3.2	1.975	0.08
4	5	13.0	−6.9	3.833	<0.01	−3.0	1.667	0.12	−3.8	2.000	0.08

the statistical analysis shows a low order of significance for differences between control and treated fish. However, the pattern of results is in general consistent with the view that these hormones produce an increased susceptibility to stimulation.

Fig. 6 summarizes the data obtained with sockeye when treated with the five different compounds. During the fourth week the reaction time was considerably shorter in every case. The slow reaction displayed by testosterone treated sockeye in the first test is inconsistent with the findings for coho (Table II), with the findings for goldfish as given above, and with findings for the swimming rates of chum salmon (10). Usually a maximum response is obtained with testosterone during this period and it seems likely that some unrecognized bias entered into this test. Metabolism tests — to be reported elsewhere — also show that the stimulating effects of testosterone on metabolism are at a peak during this period.

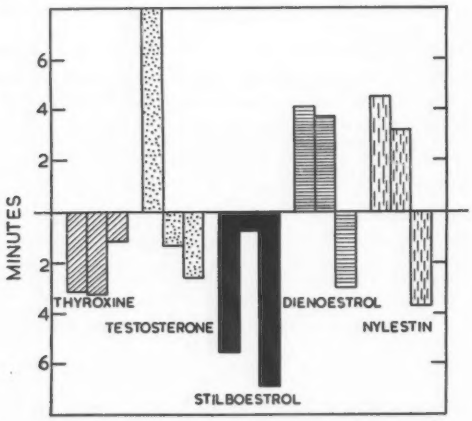


FIG. 6. Responses of sockeye salmon yearlings in the jumping test. Plotted values are deviations in minutes from the control value for the same period. The three bars grouped together give the reactions for the second, third, and fourth week of treatment in each case.

The slow initial reactions of fish immersed in dienoestrol and nylestin, on the other hand, are probably due to the physiological effects of these materials. These groups, particularly those in nylestin, showed evidence of distress throughout the period of treatment. Their movements were often erratic and they were obviously less active than the controls in the immersion baths. Other groups, at least after the first few days of treatment, were just as active and vigorous as the controls until near the end of the period.

Coho treated with thyroxine or testosterone showed reduced reaction times in five out of six tests. The inconsistency of coho treated four weeks with thyroxine was obviously related to the damaging effects of prolonged treatment with this material.

The four-week treatment with the hormones produced some evidence of damage in almost all of these experiments with salmon. In later stages of treatment the movements were often erratic even though reaction times were still shorter than those of the controls. The extreme case was that of coho smolt during the fourth week of treatment with thyroxine (Table II). These fish became obviously weakened and the same individual occasionally jumped six or eight times before crossing the dam. Controls and fish treated for shorter periods usually jump only once in order to reach the upper pool. Furthermore, during this period coho often stayed near the outlet end of the trough and did not attempt to jump. Sockeye, in contrast, showed increased activity, swimming speed and jumping although their movements were sometimes poorly coordinated. They often jumped vigorously but in various directions below the dam and did not reach the upper pool as rapidly as their evident activity would warrant.

Tables II and III show evidence of conditioning or learning on the part of the fish. The frequency of testing, as indicated in the tables, was probably great enough that the fish were progressively less startled by the capturing and transfer to the testing apparatus and thus showed a marked improvement in reaction time during succeeding tests. This was to be expected and should not bias the results since controls were in general subject to the same number of tests as the experimental groups.

After the final jumping test experiment each group was placed in rotating currents of one of the rheotaxis tubs described elsewhere (11). The control coho remained in a close group and held position in the fastest flow. Thyroxine treated coho darted about irregularly and were scattered both in fast and slower currents. Testosterone treated coho displayed vigorous positive rheotaxis moving in loose aggregates around the tub against the current. Negative rheotaxis was not observed.

Sockeye behaved less consistently in these circular currents. Positive rheotaxis was the dominant reaction but both treated and control groups occasionally darted about in the currents so that schooling was less evident than anticipated on the basis of other tests (11). The stilboestrol treated fish were the most active of all groups observed in this apparatus. They aggregated closely and showed the most strongly marked positive rheotaxis. Negative rheotaxis was not observed in any of the groups.

Discussion

The ethologist recognizes two distinct components in any complex behavior situation — the variable appetitive behavior and the uniform consummatory act. In the case of reproductive behavior, for example, fish experience an obvious surge in activity as the breeding period approaches. This results in an increased amount of movement which may be random (kineses) or directed (taxes) in accordance with the environmental situation and the genetic constitution of the species. Prolonged migrations may form some part of this

appetitive behavior. The consummatory act of spawning — with its associated activities of nest building, courtship, and so forth — will occur when the functionally mature individual eventually encounters, through its appetitive behavior, an appropriate set of stimuli or releasers. The motor patterns of these consummatory acts are genetically fixed and stereotyped in fish and the releasing situations are specific. However, the extent of the appetitive behavior, the timing of events and their intensity of expression may depend on hormones.

It is tempting to ascribe specific activities such as migration, mating, or parental care to the effects of specific hormones. There is, however, relatively scanty evidence for such speculation. Androgens have been shown to induce masculine behavior in castrates or female guppies and sword tails. However, more frequently fish treated with steroids fail to show specific sexual behavior patterns or show rather incomplete ones. The literature has been summarized (6, 9). A notable exception is the S-shaped spawning reflex in *Fundulus* (12) which can be released promptly by injection of posterior lobe extracts (15). The mechanism is independent of gonads and pituitary since it occurs in gonadectomized or hypophysectomized animals of either sex. This seems to be the best — perhaps the only real demonstration — of specific behavior patterns produced by specific hormones.

Beach (2, 3) does not believe that androgens or estrogens act as specific stimuli or organizing agents in mammals. He feels, on the contrary, that they act as chemical sensitizers which alter the responsiveness of central nervous mechanisms to stimuli. This view is consistent with the data presented here. Juvenile salmon and goldfish treated with thyroxine, androgens, or estrogens show increased locomotor activity and elevated responsiveness to at least two different types of stimulation.

The present experiments suggest that hormones may modify the intensity of appetitive behavior. If the physiological effects of thyroid and gonadal hormones are comparable to those observed in the present experiments their action will be marked in the generalized rather than the more restricted and specialized acts of appetitive behavior. Thus, thyroid and gonadal hormones, controlled by seasonal and cyclical changes in the activity of the pituitary gland, may play an important part in the spectacular appetitive behavior involved in prolonged migrations of fish. Changes in thyroid activity frequently coincide with sexual maturation (6, 9) and the stimulating effects of these associated hormones may be in part responsible for the heightened vigor apparent at the time of reproduction. This is certainly not the only way in which these hormones function. The action of steroids on the development of secondary sexual characters of fish is, for example, well established (9). However, it is here suggested that the action of thyroxine and the gonadal steroids on the generalized aspects of appetitive behavior may be added to the list of recognized functions of these hormones.

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THE DEVELOPMENT OF THE STICHOSOME AND ASSOCIATED STRUCTURES IN *TRICHINELLA SPIRALIS*[1]

By Liang-Yu Wu

Abstract

A study has been made on the development of the stichosome in *Trichinella spiralis*. Stichocytes are seen as cuboidal cells in larvae six days after infection. During the course of development, the stichocytes gradually arrange themselves in two rows above the oesophagus and elongate transversely, tending to dovetail alternately opposite each other. The number of these cells in larvae taken 13 days after infection is only half that observed in the mature larvae. Each stichocyte takes a spiral course as the elongation continues. Superficially this results in an apparent duplication of the number of rectangular cells seen. In fact, the stichosome comprises a row of about 24 to 30 unicellular, binucleate gland cells. Each has two coils and somewhat resembles a spring in form. The intestinal gland cells also originate as two cuboidal cells. These undergo separate development into two gland cells looping around the oesophageal–intestinal junction.

Introduction

The oesophagus of *Trichinella spiralis* in the adult is a long tube differentiated into two parts: a shorter anterior and a longer posterior portion. The anterior part of the oesophagus is weakly muscular. It enlarges into a bulbous structure at its posterior end, which narrows abruptly into a long, slender tube forming the posterior part of the oesophagus. Through its whole length, this fine, tubular posterior portion of the oesophagus is closely associated with a voluminous column of stichocytes (the oesophageal gland cells), which is characteristic of the Trichuroidea. This posterior tubular portion enters the pear-shaped enlargement of the anterior end of the intestine. The stichosome has been believed by previous workers to be a row of over 50 stichocytes. The present study has been undertaken to reveal some of the facts relating to the development of the stichosome.

Materials and Methods

White mice were used for experimental infections. Larvae of *T. spiralis* were obtained by artificial digestion of the muscles of infected mice. A number of mice were infected on the same day and a series of studies was made.

The first mouse was killed on the fifth day after infection and one mouse was killed at about 24-hr. intervals up to the 24th day. A small portion of the diaphragm of the infected mouse was immersed immediately in normal saline and the muscle fibers were teased apart, under a stereoscopic microscope, releasing the larvae lodged in the muscle. Most of the muscle tissue was then removed leaving the larvae behind. Hot glycerine alcohol (5% glycerine in 75% alcohol) was poured into the dish to fix the larvae. A piece of diaphragm was also teased apart in a small drop of saline on a slide which was then put in a Petri dish and hot glycerine alcohol was carefully poured onto one

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end of the slide and allowed to run gradually over the whole slide. More of the hot glycerine alcohol was then poured into the dish. The protein material, accumulated from teasing the diaphragm muscle, coagulated and held the larvae firmly on the slide, acting as a fixative. Many larvae might have been flooded and lost, but a considerable number were retained on the slide for study. Many of these preparations were made each day and were allowed to stand over night.

Since the adult worms were producing young continuously, larvae of various ages were present in the diaphragm. Therefore, the largest specimens were selected each day to represent the larvae of that age.

The specimens were stained with haematoxylin or alum cochineal and mounted in balsam or preserved in glycerine for study. Unstained glycerine specimens were also prepared.

Observations

Motile larvae, free from the embryonic membrane, were present in the uterus of the female worm five days after infection. The first larvae were found in the diaphragm six days after infection. These larvae were slightly longer than those found in the uterus of the female.

The digestive system was quite well differentiated in a same six day larva (Fig. 1). A number of irregularly cuboidal shaped cells were loosely arranged in the region where the stichosome (sts) eventually develops. Because of granules in the cells on the body wall only the outlines of a few cuboidal cells were clearly seen. These cuboidal cells are presumed to be the precursors of the stichocytes. An anterior enlargement (eo) of the muscular oesophagus was already quite well developed. The buccal stylet (not shown in drawings) was clear and was frequently seen in living specimens with its point protruding and retracting. The intestine (i) was very short, being about one-fifth of the body length.

Larval development during the next two days was slow. Specimens isolated nine days after infection (Fig. 2) began to show rapid development. The anterior oesophageal enlargement was well formed and massive at this stage. The stichocytes (stc) were much larger and more clearly outlined. At the junction between the oesophagus and the intestine, two cells (ig) were seen and were distinctly separated from the stichosome. A few large cells (gs) appearing in the body cavity were presumed to be the developing genital system.

In larvae isolated 10 days after infection (Fig. 3), the stichocytes were arranged in two rows. These cells assumed a pyramidal shape with the apices toward the median line.

Further development was shown in larvae isolated 12 days after infection (Fig. 4). Cells from each row developed transversely and dovetailed so that they were arranged more or less alternately.

In a specimen isolated 13 days after infection (Fig. 5), the stichocytes were more elongated and the apices were observed reaching the opposite border.

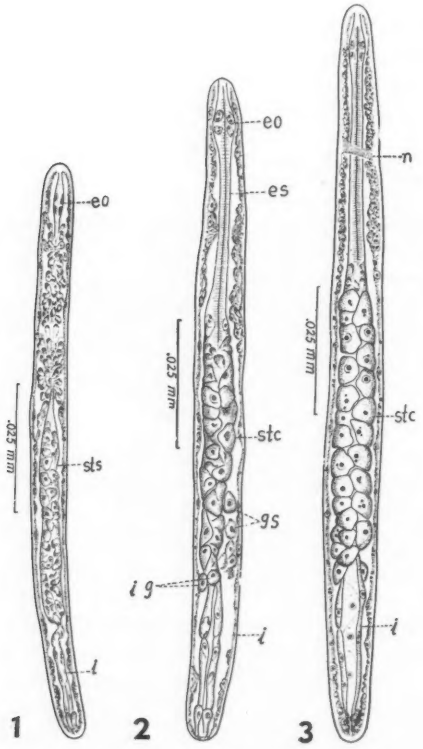
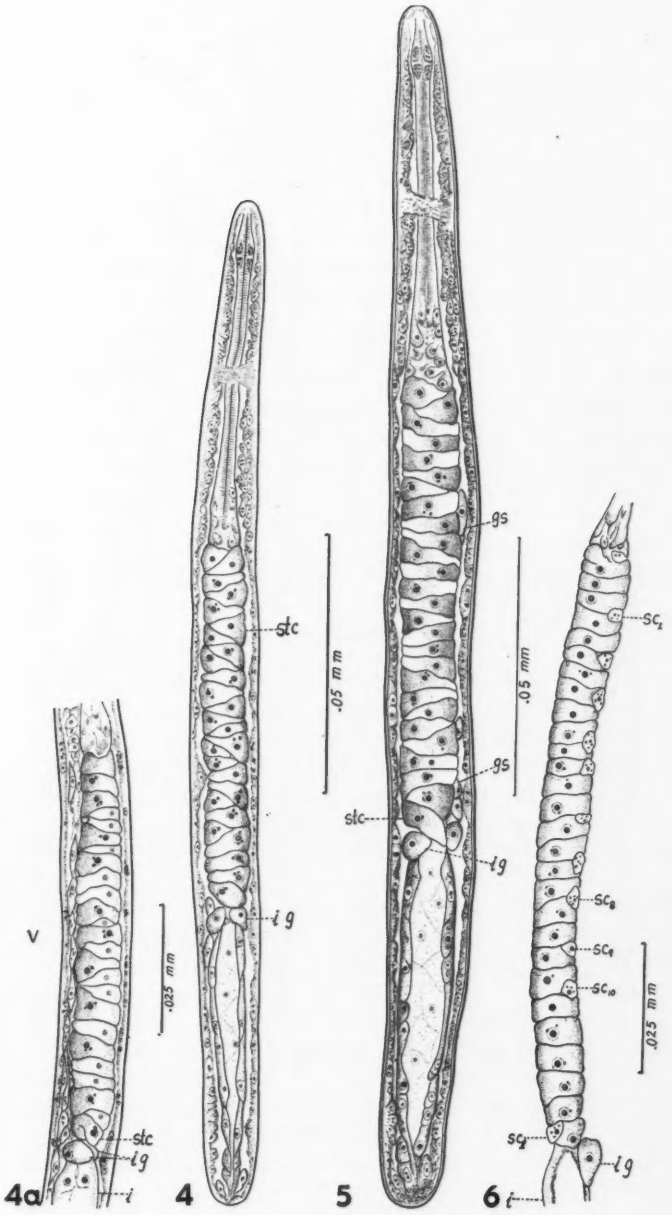


FIG. 1. Larva 6 days after infection, dorsal view.
FIG. 2. Larva 9 days after infection, dorsal view.
FIG. 3. Larva 10 days after infection, dorsal view.

Drawings were made with the aid of a camera lucida. Abbreviations: eo, enlargement of oesophagus; es, oesophagus; gs, genital system; i, intestine; ig, intestinal gland cell; n, nerve ring; sc, small cell; stc, stichocyte; stc_n, new stichocyte; sts, stichosome; v, ventral.

At this stage the stichosome appeared as a row of rectangular cells which were much broader than long. The two intestinal gland cells (ig) had become well developed, one on each side near the stichosome–intestinal junction. The genital system appeared as a chain of loosely arranged cells. The anterior oesophageal enlargement remained about the same size as that in specimens obtained nine days after infection.

FIG. 4. Larva about 12 days after infection, dorsal view.
FIG. 4*a*. A portion of larva about 12 days after infection, lateral view, left row of stichocytes shown in darker shade.
FIG. 5. Larva 13 days after infection, dorsal view.
FIG. 6. Stichosome from young trichina about 14 days after infection, lateral view; showing a series of 10 small cells (sc_1 to sc_{10}) of right side; posterior end twisted toward opposite side, showing the last small cell (sc_l) of the left side.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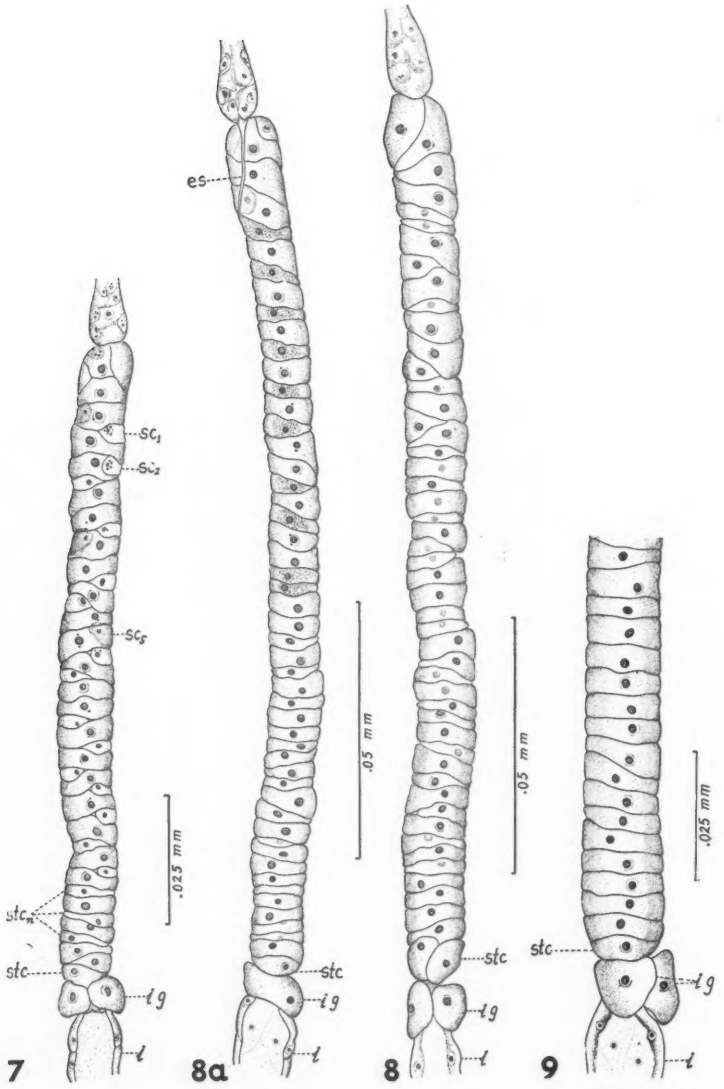

FIG. 7. Stichosome from young trichina about 15 days after infection, dorsal view; two rows of small cells interspersed between large stichocytes; sc_1, small cell underneath; three small cells at posterior end already developed into three new stichocytes (stc_n).

FIG. 8. Stichosome from young trichina about 16 days after infection, dorsal view; development of new stichocytes well advanced.

FIG. 8a. Stichosome from trichina about the same age as above, lateral view.

FIG. 9. Posterior $\frac{1}{3}$ of stichosome from trichina about 18 days after infection, dorsal view.

In a specimen taken from a mouse 14 days after infection (Fig. 6), small cells (sc) appeared along the tubular part of the oesophagus interspersed between the well-formed stichocytes on the ventral aspect. The cytoplasm of each small cell contained several deeply stained granules. Some of these granules are similar to those seen in the well-formed stichocytes in size, while others are much larger. These small cells eventually became elongated and positioned between the pre-existing stichocytes as shown in Fig. 7. The new small cells developing at the posterior end of the stichosome grew more rapidly and were seen as smaller new stichocytes (stc_n) between the larger stichocytes. The nuclei in these new cells were small. Small cells sc_1 and sc_2 near the anterior end of the stichosome contained only coarse granules of various sizes, while other cells (cells between sc_2 and stc_n) each contained a small nucleus although the size and shape varied slightly.

In slightly older specimens, taken about 16 days after infection (Fig. 8), the development of these new stichocytes was well advanced. Those on the posterior end of the stichosome appeared as narrow, rectangular cells. The total number of stichocytes varied usually between 48 to 58, each with a large nucleus. However, these nuclei were not arranged in a single row as seen in the more mature trichinae. They gradually shifted to the mid-line as development progressed.

Trichinae isolated 18 days after infection began to show a tendency to coil. The stichocytes and their nuclei shown in Fig. 9 were almost identical in size.

Discussion

The present study shows that the number of stichocytes and the number of their nuclei in the young larvae, up to 13 days after infection, is only half the number seen in the mature stage. As the original number becomes double a few days later, observations have been centered on trichinae taken 13 days after infection in order to study cytoplasmic division and particularly nuclear division in the stichocytes. In numerous specimens isolated 13 to 15 days after infection, no characteristic nuclear or cytoplasmic division has been observed.

The smaller cells (Fig. 6, sc) that appear at the bases of the first set of stichocytes on the ventral aspect eventually develop into the second set of stichocytes, each with a smaller nucleus which increases in size as the stichocyte develops.

There is a possibility that these small cells might be of separate origin, beginning to appear as small cells at the bases of the first set of stichocytes when the latter has completed its development. However, the writer inclines to the belief that these smaller cells (Figs. 6 and 7) are merely the tips of the coiled stichocytic cells of the first set and are not therefore a new set of cells at all. No nuclear or cytoplasmic division could be observed. The coarse granules seen in the smaller series of cells may be chromatin granules in the cytoplasm of the original set of stichocytic cells. In Figs. 6 and 7 a progressive formation of new nuclei seems to be in operation. Small cell sc_9 and cell sc_{10}

(Fig. 6) each contains a small nucleus, whereas small cell sc_1 to cell sc_8 have still only coarse granules. In Fig. 7 small cell sc_1 and cell sc_2 still contain coarse granules, whereas the rest of the small cells each has a small nucleus. These new nuclei gradually increase in size and become identical with the nuclei in the first set of the stichocytes as seen in Fig. 9.

Accordingly it is suggested that each stichocyte is a unicellular gland with two nuclei. Each originates as a cuboidal cell. The stichocytes elongate transversely, tending to dovetail alternately. As development goes on, each cell increases in size and length. The tip of each elongating gland cell curves ventrally downward, then upwards again on the opposite side, appearing deceptively as a separate cell (Fig. 6 sc). The elongation continues until the apex appears as a small rectangular cell poised between two stichocytes. Thus each cell takes a spiral course as it elongates. The stichocytes of the "second set" are merely the distal halves of the stichocytes of the "first set". This development occurs dorsally to the oesophagus.

Chitwood (1) found that both the oesophagus and the stichosome are surrounded by separate membranes which may at times join each other. In studying dissected adult specimens the oesophagus has never been found free from the stichosome. This also suggests that the oesophagus and the stichosome are closely associated. In young larvae, the oesophagus appeared as a straight slender tube lying on one side of the stichosome. The twisting of the oesophagus from one side to another, so characteristic in the mature trichinae, was first noticed in some specimens taken about 18 or 19 days after infection.

In *Trichuris* it has been established by Chitwood and Chitwood (3) that each stichocyte has a separate orifice reached or formed by a tube through the oesophageal wall. These orifices alternate (2, 3). These authors also suggested "that the orifices in the adult tend to alternate. It seems reasonable to assume that the single row stichosome of *Trichuris* is a later evolutionary development from a double row of stichocytes". Since ontogeny tends to recapitulate phylogeny, the result of the present study on the development of the stichosome in *Trichinella* might verify their opinion concerning *Trichuris*.

There is a well-developed enlargement on the anterior part of the muscular oesophagus in the early stage suggesting that it may be an active sucking structure during the very young stage. This structure disappears in mature muscle trichinae.

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CONTENTS OF VOLUME 33

Anderson, R. C. *Ornithofilaria algonquinensis* n. sp. from *Hirundo erythrogaster* with a revision of the genera *Paramicipsella* Chow, 1939 emend. Chabaud and Choquet, 1953 and *Ornithofilaria* Gönnert, 1937, 107.

Banfield, A. W. F. A provisional life table for the Barren Ground caribou, 143.

Barlow, C. A. The fecundity of *Aedes hexodontus* Dyar (Culicidae) in the laboratory, 420.

Belcourt, J. See Bellemare, E. R., 175.

Bellemare, E. R. and **Belcourt, J.** Influence du dérivé cyanuré du DDT sur le rythme cardiaque de *Periplaneta americana* (L.), 175.

Bendell, J. F. Disease as a control of a population of blue grouse, *Dendragapus obscurus fuliginosus* (Ridgway), 195.

Bennett, G. F. Studies on *Cuterebra emasculator* Fitch 1856 (Diptera: Cuterebridae) and a discussion of the status of the genus *Cephenemyia* Ltr. 1818, 75.

Brown, J. H. Colorado tick fever in Alberta, 389.

Cameron, C. J. See Trussell, P. C., 327.

Choquette, L. P. E. The life history of the nematode *Metabronema salvelini* (Fujita, 1920) parasitic in the speckled trout, *Salvelinus fontinalis* (Mitchill), in Quebec, 1.

Church, N. S. Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. (Hymenoptera: Cephidae), 339.

Edwards, D. K. and **Jansch, M. E.** Two new species of dermatitis producing schistosome cercariae from Cultus Lake, British Columbia, 182.

Fulton, C. O. See Trussell, P. C., 327.

Goodall, R. G. See Hoar, W. S., 428.

Greer, B. A. See Trussell, P. C., 327.

Heimpel, A. M. The pH in the gut and blood of the larch sawfly, *Pristiphora erichsonii* (Htg.), and other insects with reference to the pathogenicity of *Bacillus cereus* Fr. and Fr., 99.

Investigations of the mode of action of strains of *Bacillus cereus* Fr. and Fr. pathogenic for the larch sawfly, *Pristiphora erichsonii* (Htg.), 311.

Hoar, W. S. See Stringer, G. E., 148.

Hoar, W. S., Keenleyside, M. H. A., and **Goodall, R. G.** The effects of thyroxine and gonadal steroids on the activity of salmon and goldfish, 428.

Holling, C. S. The selection by certain small mammals of dead, parasitized, and healthy prepupae of the European pine sawfly, *Neodiprion sertifer* (Geoff.), 404.

Ives, W. G. H. Estimation of egg populations of the larch sawfly, *Pristiphora erichsonii* (Htg.), 370.

Jansch, M. E. See Edwards, D. K., 182.

Keenleyside, M. H. A. See Hoar, W. S., 428.

Mackay, M. R. Cytology and parthenogenesis of the wheat stem sawfly, *Cephus cinctus* Nort. (Hymenoptera: Cephidae), 161.

Mason, W. R. M. A revision of the Nearctic Cteniscini (Hymenoptera: Ichneumonidae). I. *Eudiaborus* Kerrich and a new genus, 18.

Miller, C. A. A technique for assessing spruce budworm larval mortality caused by parasites, 5.

Morris, R. F. The development of sampling techniques for forest insect defoliators, with particular reference to the spruce budworm, 225.

Mulvey, R. H. Oogenesis in several free-living and plant-parasitic nematodes, 295.

Salt, R. W. Extent of ice formation in frozen tissues, and a new method for its measurement, 391.

Stringer, G. E. and **Hoar, W. S.** Aggressive behavior of underyearling Kamloops trout, 148.

Trussell, P. C., Fulton, C. O., Cameron, C. J., and **Greer, B. A.** Marine borer studies: Evaluation of toxicants, 327.

Wolfgang, R. W. Studies of the trematode *Stephanostomum baccatum* (Nicoll, 1907). III. Its life cycle, 113.

Studies of the trematode *Stephanostomum baccatum* (Nicoll, 1907). IV. The variation of the adult morphology and the taxonomy of the genus, 129.

Wu, Liang-Yu. The development of the stichosome and associated structures in *Trichinella spiralis*, 440.

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Contents

Page

Colorado Tick Fever in Alberta—*John H. Brown* - - - - - - - 389

Extent of Ice Formation in Frozen Tissues, and a New Method for Its Measurement—*R. W. Salt* - - - - - - - - - - - - 391

The Selection by Certain Small Mammals of Dead, Parasitized, and Healthy Prepupae of the European Pine Sawfly, *Neodiprion sertifer* (Geoff.)—*C. S. Holling* - - - - - - - - - - - - - - - 404

The Fecundity of *Aedes hexodontus* Dyar (Culicidae) in the Laboratory—*C. A. Barlow* - - - - - - - - - - - - - - - 420

The Effects of Thyroxine and Gonadal Steroids on the Activity of Salmon and Goldfish—*W. S. Hoar, M. H. A. Keenleyside, and R. G. Goodall* - - 428

The Development of the Stichosome and Associated Structures in *Trichinella spiralis*—*Liang-Yu Wu* - - - - - - - - - - - - - 440

Contents of Volume 33 - - - - - - - - - - - - 447